Towards hES cell-based therapy of Epidermolysis Bullosa

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ABSTRACT

Regenerative medicine offers great hope for therapeutic innovation in many diseases, including those with defective skin, such as the group of inherited blistering skin diseases known as epidermolysis bullosa. The work in this thesis addresses some of the important pre-clinical approaches to generating keratinocytes (the main cell of the outer skin layer, epidermis) from pluripotent stem cells (human embryonic stem cells, hESCs). Undifferentiated hESCs were exposed to a complex microenvironment in organotypic cultures (to mimic conditions for epidermal stem cell development and maintenance); this yielded epidermis-like structures which stained positively for basal keratin 14 and several extracellular matrix molecules, although a stratified epidermis was not produced. To refine the protocol, hESCs were differentiated to an epidermal cell fate in monolayer cultures *in vitro* prior to subjecting them to organotypic culture. The most efficient technique involved culture of hESCs on native de-cellularized extracellular matrix produced by normal human dermal fibroblasts as a substrate for differentiation. Large epithelial-like sheets positive for keratin 14 and p63 expression were observed. On subculture, these cells retained their morphological and immunocytochemical characteristics for up to 5 passages and could be successfully cryo-preserved and recovered. Overall, this study explored the potential of pluripotent stem cells as a source of epidermal progenitors, with results that provide proof-of-concept for future cell therapy innovations in defective skin diseases such as epidermolysis bullosa.

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ABBREVIATIONS

ADMP	anti-dorsalising morphogenetic factor
A-MSC	adipose tissue-derived MSC
ATRA	all-trans-retinoic acid
BCA	bicinchoninic acid
bFGF	basic FGF
BLAST	basic local alignment search tool
BLRCM	BRL Conditioned Medium
BM	basement membrane
BMDF	bone marrow-derived fibroblast
BMDK	bone marrow-derived keratinocyte
BM-HSC	bone marrow-derived-HSC
BM-MSC	bone marrow-derived MSC
BMP	bone morphogenetic protein
BMZ	basement membrane zone
BPAg	bullous pemphigoid antigen
BRL	buffalo rat liver
BSA	bovine serum albumin
cca	*circa*
cDNA	complementary DNA
CFE	colony forming efficiency
cPFT-α	cyclic PFT-α
CPP	cell penetrating protein
Ct	threshold cycle
d	days
DAPI	4',6-diamidino-2-phenylindole

DED	de-epidermized dermis
DEJ	dermal-epidermal junction
DKSFM	defined keratinocyte serum-free medium
DMEM	Dulbecco’s modified Eagle medium
D-MSC	dermal MSC
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DSC	desmocollin
DSG	desmoglein
EB	epidermolysis bullosa
E-BMT	embryonic bone marrow transplantation
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EHS	Engelbreth-Holm-Swarm
EMT	epithelial-mesenchyme transition
EpSC	epidermal stem cell
ESC	embryonic stem cell
ES-FCS	embryonic stem cell-tested foetal calf serum
FACS	fluorescent-associated cell sorting
FBS	foetal bovine serum
FC buffer	flow cytometry buffer
FGF	fibroblast growth factor
GMP	good manufacturing practice
HBSS	Hanks' balanced salt solution
HDAC	histone deacetylase
HDF	human dermal fibroblast
HDF-ECM	HDF-derived ECM

hESC	human embryonic stem cell
HFEA	human fertilisation and embryology authority
HFF	human foreskin fibroblast
HIV-TAT	human immunodeficiency virus transactivator of transcription
HMTase	histone methyl transferase
HSC	haematopoietic stem cell
i.e.	*Id est*
ICM	inner cell mass
iPSC	induced pluripotent stem cell
IVF	in vitro fertilisation
Klf4	krüppel-like factor 4
Ko-SR	KnockOut™ serum replacement
LG domain	laminin globular domain
M	mole
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
min	minutes
ml	millilitre
mm	millimetre
mM	millimole
µl	micro litre
µM	micro mole
MSC	mesenchymal stem cell
NGS	normal goat serum
NHK	normal human keratinocyte
nM	nanomole
NNEA	non-essential amino acids
NOD-SCID	non-obese diabetic/severe combined immunodeficiency
Oct	octamer

OD	optical density
PAS	Periodic acid-Schiff
PA6 CM	PA6 conditioned medium
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PFT-α	pifithrin-α
PG	proteoglycan
PGD	pre-implantation genetic diagnosis
pKPC	putative keratinocyte precursors
P-MSC	placenta-derived MSC
RAR	retinoic acid receptor
RDEB	recessive dystrophic EB
s	seconds
SD	standard deviation
SDIA	stromal-derived inducing activity
SEM	standard error of the mean
SKP	skin-derived precursor
SLO	streptolysin O
Sox	sex determining region Y (SRY)-box
SSEA	stage specific embryonic antigen
TE	trophoectoderm
TGase	transglutaminase
TGFβ	transforming growth factor β
TRA	tumour recognition antigen
UCB-HSC	umbilical cord blood-derived HSC
UC-MSC	umbilical cord-derived MSC
v/v	volume : volume
VEGF	vascular endothelial growth factor

VPA	valproic acid
w/v	weight : volume

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DECLARATION

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university.

ASSOCIATED PUBLICATIONS

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CHAPTER 1 GENERAL INTRODUCTION

1.1 OVERVIEW OF SKIN FUNCTION

Skin provides a defensive barrier against pathogens and environmental stresses, such as dehydration and temperature fluctuations, as well as mechanical resistance to trauma. The epidermis is a highly specialized epithelium that has evolved to provide a stable environmental barrier to prevent dehydration, resist mechanical and chemical stress and participate in immune responses. This barrier is generated, in part, by keratinocytes, the main cells of the epidermis, which form an adhesive network organized into multiple layers, or 'strata' (Fuchs and Raghavan, 2002, Candi et al., 2005) (Figure 1). However, despite its remarkable stability, epidermis is amongst the most dynamic tissues in human body, which allows for tissue regeneration every 3-4 weeks. This function is supported by epidermal stem cells, which are responsible for constantly renewing the epidermis and generating the skin barrier.

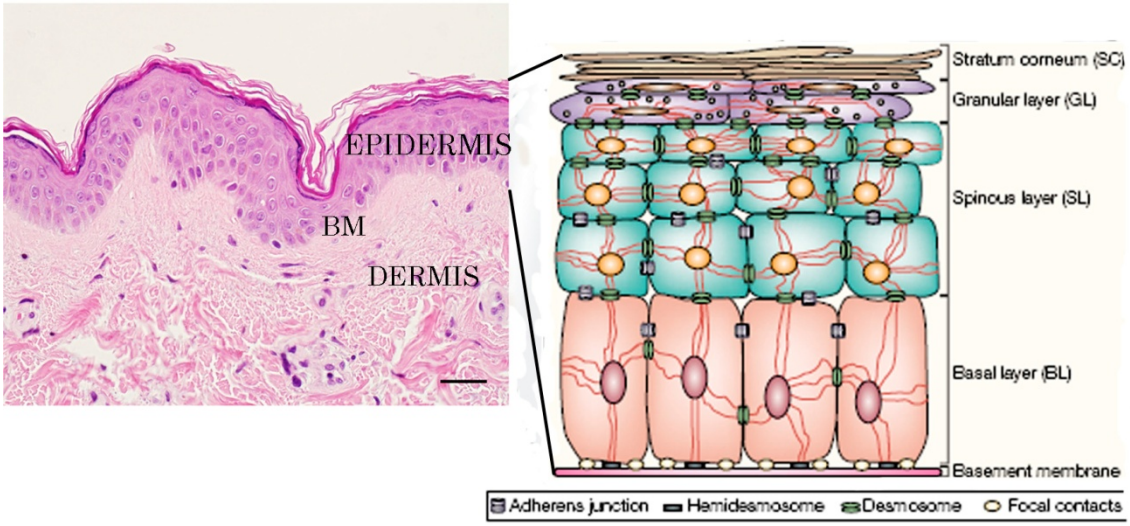

Figure 1 Structure of epidermis within the skin.
Skin is composed of two layers: the epidermis and dermis, separated by a basement membrane (BM). The epidermis is a stratified squamous epithelia that is composed of several cell layers. Attached to the BM is the basal layer (BL), consisting of highly proliferating cells. The BL stratifies to give rise to differentiated cell layers of the spinous layer (SL), granular layer (GL) and the stratum corneum (SC). Modified from (Fuchs and Raghavan, 2002).

1.2 STEM CELLS WITHIN THE SKIN

Skin harbours several adult stem cell populations, each with a specific location and molecular characteristics; these are summarized in Figure 2.

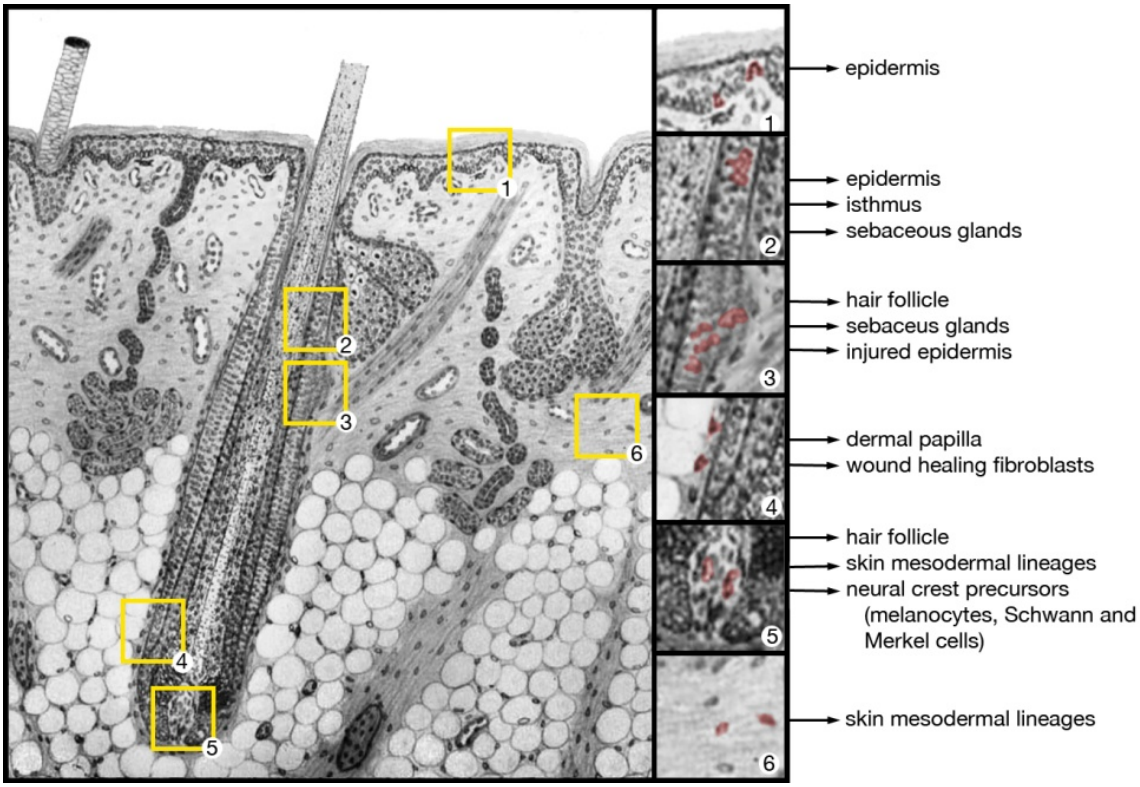

Figure 2 Location of adult stem cells in normal human skin and their role in homeostasis.
Inserts indicate various locations within skin that harbour stem cells, arrows on the right indicate the contribution of a particular stem cell population to maintenance of skin homeostasis 1) Interfollicular epidermal stem cells; 2) Bulge epidermal stem cells; 3) Isthmus epidermal stem cells; 4) Hair follicle dermal-sheath stem cells; 5) Skin-derived precursors; 6) Dermal mesenchymal stem cells.

1.2.1 Epidermal stem cells

Adult stem cells can be defined by certain criteria: they are relatively undifferentiated, both morphologically and functionally; they provide a self-renewing source for long-term tissue maintenance; and they are normally

slow-cycling *in vivo* but possess high proliferative potential which can be triggered in response to tissue injury and to certain growth stimuli. In addition, adult stem cells are normally located in a sheltered microenvironment, referred to as a "niche", which contributes to, and regulates stem cell activity and behaviour (Lavker and Sun, 1982, Potten and Loeffler, 1990). Slow-cycling stem cells within skin have been identified experimentally as "label-retaining cells" (Bickenbach, 1981, Potten, 1987). Using this approach, two populations of epidermal stem cells (EpSCs) have been identified: the first was localized to the basal layer of epidermis; the second was shown to reside in the bulge area of hair follicles. More recently, a third population of EpSCs was localized to the upper isthmus, a region between the bulge and the sebaceous gland.

1.2.1.1 Interfollicular EpSCs.

In 1987, the heterogeneity of human EpSCs was demonstrated when three different types of clonal expansion were observed in human primary keratinocytes culture: holoclone, paraclone and meroclone (Barrandon and Green, 1987). The holoclone, the smallest colony-founding cell with the highest proliferative capacity, is considered to be the prototypic EpSC (Pellegrini et al., 1999, Jones and Watt, 1993). A single holoclone can double enough times within a few weeks to produce an area equivalent to the skin surface of an adult (~$8x10^{10}$ cells). Subsequent studies have shown that human EpSCs firmly adhere to basement membrane and represent a minor subpopulation (~2–7% of basal keratinocytes) of relatively quiescent cells

(Bickenbach, 1981, Li et al., 1998). These cells are mitotically active and contribute to the homeostasis of the epidermis by their constant proliferation into rapidly dividing differentiated progeny (Alonso and Fuchs, 2003). The first progeny of stem cell division has been termed transient amplifying cells, which have a limited proliferative potential, yet share many molecular markers with their parent cell population (Jones and Watt, 1993). Some EpSCs may give rise to daughter cells that lack attachment to BM and which are already committed to terminal differentiation, a process known as asymmetrical cell division (Blanpain and Fuchs, 2009). Comprehensive efforts have been made to identify and isolate a pure population of both mouse and human EpSCs, including combined use of cell kinetic analysis and fluorescent-associated cell sorting (FACS) *in vivo* (Tani et al., 2000, Li et al., 1998). EpSCs display high levels of α6 integrin, and low-to-undetectable expression of the transferrin receptor, CD71. Use of FACS, however, introduces some limitations as cell viability is partially compromised. As a further approach, adhesion-selection methods can be used (Radu et al., 2002). These have shown that human EpSCs can be directly enriched from keratinocytes based on their rapid adherence to the ß1-integrin ligand collagen type IV, and by their small size (5–7μm) (Chino et al., 2008). Despite the existence of enrichment approaches mentioned above, isolation of a pure and viable population of EpSCs has not yet been achieved. A number of other molecular markers have been suggested as putative EpSCs markers, including p63, (Pellegrini et al., 2001), a homologue of the p53 tumour suppressor gene which exists as six different isoforms under the control of two distinct promoters and three different

alternative splicings leading to the production of ΔN- or TA-p63, and α, ß or γ isoforms, respectively (Yang et al., 1998). The involvement of p63 has been implicated in both the development and maintenance of stratified epithelia in mice (Mills et al., 1999, Yang et al., 1999). Expression of p63 was shown to be indispensible for the initiation of epidermal stratification when the exogenous expression of p63 in single-layered lung keratin 8/keratin 18-positive epithelial cells was able to initiate a stratification program, with formation of keratin 14-positive cells (Koster et al., 2004). Accordingly, absence of p63 results in premature termination of stem cell proliferation and failure to form stratified epithelium (Senoo et al., 2007). Other possible markers include P-glycoprotein, a multidrug resistance pump that mediates Hoechst 33342 dye exclusion, in murine keratinocytes (Sleeman et al., 2000), while all keratins 15 (Lyle et al., 1998) and 19 (Stasiak et al., 1989) and raised levels of ß catenin (Zhu and Watt, 1999) have been implicated in human EpSCs.

1.2.1.2 Bulge EpSCs.

The hair follicle contains an outer root sheath that is contiguous with the epidermis, an inner root sheath and a hair shaft. The matrix surrounding the dermal papilla, in the hair root, contains actively dividing, relatively undifferentiated cells and therefore represents a source of mesenchymal cells that are essential for follicle formation. In mice, the bulge region, located below the sebaceous gland, is a niche for multipotent EpSCs (Tumbar et al., 2004, Morris et al., 2004, Blanpain et al., 2004).

Subsets of these follicle-derived multipotent stem cells can be activated during injury and migrate out of hair follicles to the site of a wound to repair the damaged epithelium (Ito et al., 2005, Nowak et al., 2008); however, they contribute little to the homeostasis of intact epidermis. Instead these hair-follicle stem cells mostly contribute to the growth of follicles themselves and the sebaceous gland (Morris et al., 2004, Levy et al., 2005). In addition, the bulge contains melanocyte stem cells (Steingrimsson et al., 2005). Furthermore, nestin-positive progenitor cells have been identified within the bulge region; these cells can differentiate into neurons (Amoh et al., 2008), glia, keratinocytes, smooth muscle cells, melanocytes and blood vessels (Amoh et al., 2005a, Amoh et al., 2005b).

1.2.1.3 Isthmus EpSCs.

A further putative EpSC population has recently been identified in the upper isthmus of the murine hair follicle, a region between the bulge and the sebaceous gland. These cells are positive for mTS24 and Lrig1 marker proteins (Nijhof et al., 2006), and can differentiate into all epidermal cell lineages after transplantation (Jensen et al., 2008). Recently, Lrg6-expressing cells in the isthmus that can generate both hair follicles and sebaceous glands during development have been identified (Snippert et al., 2010). Furthermore, Lrg-6 cells may also contribute to some cells of the interfollicular epidermis. Like isthmus cells, Lrg6-expressing cells transplanted into immunodeficient mice give rise to all epidermal cell

lineages, and, like bulge cells, can be activated in injury to aid wound repair (Snippert et al., 2010).

1.2.2 Dermal Stem Cells

The dermis also contains several adult stem cell populations.

1.2.3.1 Hair-follicle dermal-sheath stem cells.

These cells contribute to the formation of dermal papilla cells and wound-healing fibroblasts (Reynolds et al., 1999, Gharzi et al., 2003). One of the main advantages of hair-follicle-derived stem cells is their unique immunological profile: the virtual absence of major histocompatibility complex (MHC) class I expression and low numbers of associated immune cells (Paus et al., 2003). Furthermore, the presence of resident mesenchymal stem-cell-like cells has been reported in both rodent and human dermis (Jahoda and Reynolds, 2001). These cells not only contribute to mesodermal derivates of the dermis, but are also able to differentiate along mesenchymal stem cell pathways to produce adipocytes, chondrocytes, haematopoietic and neuronal precursors – both *in vitro* and *in vivo* (Gharzi et al., 2003).

1.2.3.2 Skin-derived precursors.

The hair and whisker follicle dermal papillae have also been shown to harbour multipotent nestin- and fibronectin-positive precursors (Biernaskie et al., 2009, Toma et al., 2001). These cells, termed skin-derived precursors (SKPs), are distinct from mesenchymal stem cells and their capacity to differentiate into cells of neuroectodermal and mesodermal lineage was shown in both mice and human studies (Toma et al., 2001, Buranasinsup et al., 2006). SKPs share many characteristics with embryonic neural crest stem cells, including neural-crest-like differentiation potential *in vitro* and *in vivo*, and for whisker follicle dermal papillae, the cells are neural-crest-derived (Fernandes et al., 2004).

1.2.3.3 Dermal mesenchymal stem cells (D-MSCs).

These cells can be obtained by adherent culture from low-temperature preserved human foreskin biopsies (Bartsch et al., 2005). The group has shown that D-MSCs are able differentiate into mesodermal lineages including adipocytes, osteocytes and myocytes. Recently, dermal fibroblasts themselves have been dissected based on clonal analysis of their differentiation potential (Chen et al., 2007). This study showed that nestin-negative, vimentin-positive fibroblasts may represent a novel type of multipotent adult stem cells in human dermis. In addition, another multipotent MSC population has been isolated from within a population of human skin fibroblasts (Kuroda et al., 2010). These cells, which the authors

termed Muse cells, are positive for pluripotency markers and able to differentiate into all three germ layers.

The skin stem cell populations described co-operate together to replenish the epidermis and replace keratinocytes that are lost either through normal differentiation and tissue turnover, or through environmental insults, thereby maintaining skin integrity. However, this function can be severely compromised in certain skin conditions such as epidermolysis bullosa (EB).

1.3 EPIDERMOLYSIS BULLOSA

EB is a group of blistering skin disorders resulting from mutations in one of 15 different genes encoding protein components of the cutaneous basement membrane zone (BMZ), keratinocyte or cell-cell adhesion complexes (Intong and Murrell, 2012). These mutations affect the organization of BMZ, causing the epidermis to separate from the dermis. The site of cleavage in the dermal-epidermal junction (DEJ) is dependent on which of the genes is defective (Figure 3). EB is classed according to the site of cleavage into four subtypes: EB simplex, dystrophic EB, junctional EB, and Kindler syndrome (Verneris et al., 2009, Fine et al., 2008). In EB simplex tissue separation is intraepidermal, the lamina lucida cleavage occurs in junctional EB, whereas in dystrophic forms of EB blistering is found below the lamina densa.

Recessive dystrophic EB (RDEB) is one of the most severe forms of EB. The skin of RDEB patients can blister with a slightest touch and wound healing is severely compromised, causing debilitating scarring. In addition, mucosal

blistering and scarring is very common. The skin integrity is severely compromised, causing frequent infections due to loss of the protective function. The leading cause of death of patients with RDEB is squamous cell carcinomas (McGrath et al., 1991).

The primary pathology of RDEB involves reduced or absent expression of the anchoring fibril protein type VII collagen at the DEJ leading to sub-epidermal blistering (Bruckner-Tuderman et al., 1989, Hovnanian et al., 1992, McGrath et al., 1993a). In RDEB, the level of blistering is below the BMZ which is likely to deplete the pool of interfollicular EpSCs, thereby disrupting skin homeostasis. Currently, there is no effective treatment for RDEB, although progress is being made in developing new therapies, including cell therapy approaches (Uitto, 2011, Uitto et al., 2012). It is important to remember that for a genetic skin disease, such as RDEB, correction of the underlying genetic defect or sustained allogeneic transplantation from healthy donors would be required in order to achieve successful cell therapy. Both keratinocytes and fibroblasts can synthesize and secrete type VII collagen (Stanley et al., 1985), and therefore both cell types might be beneficial for restoring the integrity of BMZ in RDEB. However, due to the constant turnover of skin, the ideal cell therapy would have to be aimed at resident skin stem cells in order to have a long-term sustainable therapeutic effect; alternative approaches in somatic cells might only provide partial and/or temporal relief.

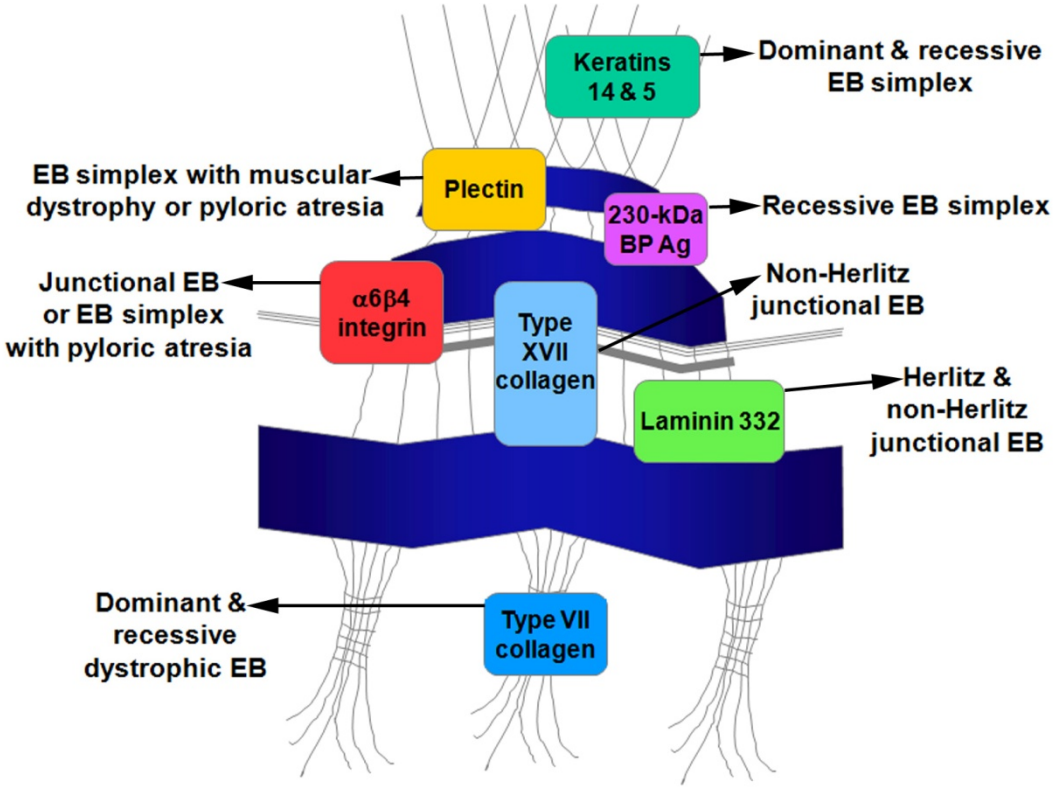

Figure 3 The classification of Epidermolysis Bullosa.
Classification is based on the localization of the defective gene within the DEJ. EB- Epidermolysis Bullosa; BP Ag- Bullous Pemphigoid antigen

1.4 POTENTIAL ADULT STEM CELL SOURCES FOR SKIN REGENERATIVE THERAPY

With respect to treatment for skin fragility diseases, such as RDEB, several stem cell types have been studied and explored. These can be broadly divided into resident skin stem cells (Table 1) and stem cells from tissues other than the skin (Table 2).

Cell type	Source	Therapeutic potential	Advantages	Disadvantages
Epidermal Stem Cells	Interfollicular EpSCs	Transplantation of genetically corrected EpSCs	Autologous source. Permanent correction of epidermis	Difficult to isolate. Endogenous pool is likely to be reduced. Genetic correction required
	Bulge EpSCs			The extent of contribution to epidermis is unclear
	Isthmus EpSCs			
Dermal Stem Cells	Hair follicle dermal-sheath stem cells	Type VII collagen expressing fibroblast	Unique immunological profile allows for allogeneic transplantation	Difficult to isolate. Unlikely to contribute to epidermal repair
	Skin-derived precursors			
	Dermal-MSCs	Potential source of EpSCs	Autologous source. Easily accessible	Genetic correction required. Differentiation potential unknown

Table 1 Summary of different stem cell types within the skin that that could potentially be used in skin regenerative therapy.

Cell type	Source	Therapeutic potential	Advantages	Disadvantages
Mesenchymal Stem Cells	BM-MSC	Allogeneic transplant/ Embryonic-BMT	Type VII collagen production by BMDFs. Potential *in utero* treatment. Skin regenerative effects via paracrine action	BMDFs preferentially deposited to hair follicles. Might not be useful in older patients. Unlikely to contribute to epidermal repair
	A-MSC	Allogeneic transplant	Readily accessible. Skin regenerative effects via paracrine action	Mostly wound healing effects. Only partial/temporal amelioration
	UC-MSC	Allogeneic transplant	Abundance of material. Can be stored for a long time	Low numbers. Only partial/temporal amelioration
	P-MSC	Allogeneic transplant	Unlimited source of tissue. Non-invasive isolation	May have tumourogenic properties. Low numbers from a single placenta. Only partial/temporal amelioration
Haematopoietic stem cells	BM-HSC and UC-HSC	Allogeneic Bone marrow/umbilical cord blood infusion	Induce type VII collagen production. Native immune status. Contribute to revascularization. Paracrine effects on resident stem cells	Limited capacity for self-renewal and proliferation. Mechanisms not clear. Likely to provide only partial/temporal amelioration
Pluripotent Stem Cells	hESCs	Differentiation protocols to produce EpSC-enriched population	Indefinite capacity for self-renewal and differentiation. ESC-derived EpSCs would provide permanent restoration	Ethical consideration. Safety concerns regarding teratoma formation
	iPSCs	Generation of genetically-corrected EpSCs for transplantation. Generation of RDEB-specific iPSC lines	Potential patient-specific cell therapy. Disease model from RDEB-cell lines	Safety concerns regarding the use of viral vectors. Safety concerns regarding teratoma formation

Table 2 Summary of different stem cell types from tissues other than the skin that that could potentially be used in skin regenerative therapy.

1.4.1 Epidermal stem cells for skin regenerative therapy

Despite numerous studies, the extent to which stem cells from the bulge region and the isthmus could contribute to epidermal regeneration remains unclear, thus impeding their potential application in cell therapy for EB. On the other hand, the importance of interfollicular stem cells in skin regenerative therapy has been confirmed by keratinocyte-mediated cell therapy in the treatment of full-thickness burns (Pellegrini et al., 1998). The first approaches for skin regeneration were mostly concerned with wound repair and utilized autologous (Simon et al., 1995) or allogeneic grafting systems, such as commercially available Apigraf® (Veves et al., 2001). Specifically, in RDEB skin grafting has been performed with cultured keratinocytes allografts (McGrath et al., 1993b), autologous cultured keratinocytes (Verplancke et al., 1997), allogeneic composite cultured skin grafts (Eisenberg and Llewelyn, 1998, Fivenson et al., 2003) and autologous epidermal grafts (Wollina et al., 2001). However, it is now clear that the long-term function of the graft is limited by the fact that grafted keratinocytes are on the path to terminal differentiation due to prolonged culture *in vitro*. It has been demonstrated that progenitor cells are superior over differentiated keratinocytes in the generation of engineered skin (Pellegrini et al., 1999). To date thousands of victims of third-degree burns have been treated by the means of cultured EpSCs as confirmed by follow-up studies over a 20 year period (De Luca et al., 2006), thus confirming the capacity of EpSCs to maintain the clonogenic potential, growth rate and long-term proliferation potential in the graft.

Despite the proof of their self-renewal and multipotency, EpSCs remain difficult to isolate due to the lack of well-defined markers thereby impeding their application for skin regenerative therapies. Recently, a first-in-man clinical trial in patient with non-Herlitz junctional EB showed a full functional correction of the disease through *ex vivo* correction of the underlying laminin-332 defect in autologous EpSCs (Mavilio et al., 2006). It should be noted, however, the authors were unable to obtain clonogenic and holoclone-forming cells from most of the patient's skin. Increasing evidence suggests that in EB patients there is a depletion or a potential exhaustion of the endogenous EpSC population, thus implying that it would be a major technical challenge to obtain a sufficient amount of autologous EpSCs for further genetic correction and therapy. Therefore, alternative sources of stem cells should be explored.

1.4.2 Dermal stem cells for skin regenerative therapy

Dermal stem cells might prove to be useful in restoring the normal function of type VII collagen in RDEB. It has been shown that gene-corrected RDEB fibroblasts and normal human fibroblasts alone could restore type VII collagen expression at the BMZ *in vivo* (Woodley et al., 2003). Furthermore, a recent clinical has shown that intradermal injections of allogeneic fibroblasts can potentially benefit patients with RDEB (Wong et al., 2008). The main drawback of this approach is that the presence of multiple wounds in RDEB would require numerous intradermal injections which are both painful and difficult to administer. However, multiple injections can be

potentially avoided if dermal stem cells are utilized instead. Nevertheless, these remain difficult to isolate and are likely to represent a very rare population within the dermis. Moreover, the main defect in EB lies within the epidermis, strongly suggesting the importance of restoring the epidermal compartment of the skin.

On the other hand, multipotent MSCs within dermis might provide a potential source for generating EpSCs. In addition, they have a practical advantage of easy accessibility compared to other MSCs. However, knowledge on resident skin MSC biology remains limited and the extent of their differentiation potential into keratinocytes remains unclear, thus for time being, alternative sources of multipotent progenitors should be considered.

1.4.2 Mesenchymal Stem Cells (MSCs) for skin regenerative therapy

1.4.2.1 Bone marrow-derived MSCs (BM-MSCs)

These cells participate in the regeneration of mesenchymal lineages by providing fibroblast-like cells in the dermis and the number of these cells increases in skin wounding (Fathke et al., 2004, Ishii et al., 2005). Furthermore, several studies have shown that MSCs participate in the regeneration of skin in cutaneous wounds (Sasaki et al., 2008, Wu et al., 2007, Mansilla et al., 2005, Deng et al., 2005, Badiavas and Falanga, 2003, Badiavas et al., 2003). Notably, BM-MSCs have been shown to

transdifferentiate into keratin-14-positive cells and into keratinocytes, which are recruited to epidermis, hair follicles, and sebaceous glands (Sasaki et al., 2008, Krause et al., 2001, Kataoka et al., 2003, Inokuma et al., 2006). However, these BM-derived keratinocytes are rarely detected, and comprise only ≈0.0001–0.0003% of all keratinocytes in the new epidermis (Fan et al., 2006). Nevertheless, very recently, a specific subset of BM-MSCs with epithelial differentiation potential has been identified (Tamai et al., 2011). These platelet-derived growth factor receptor α-positive (Lin−/PDGFRα+) BM-MSCs were mobilized in response to elevated high mobility group box 1 (HMGB1) levels in serum following skin grafting, and were shown to significantly contribute to epidermal regeneration.

A further mechanism by which BM-MSCs can promote wound healing is through release of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and angiopoietin-1 (Wu et al., 2007). The use of an autologous BM-MSC/artificial dermis composite graft has also been shown to augment wound healing (Yoshikawa et al., 2008). Other findings demonstrate that BM-MSCs can also contribute to repair of skin appendages (Li et al., 2006).

1.4.2.2 Adipose tissue-derived MSCs (A-MSC)

Subcutaneous adipose tissue represents an alternative source of MSCs and is readily accessible from routinely performed liposuction or other plastic surgery procedures. Thus, A-MSCs have an advantage over BM-MSCs in terms of ease of tissue availability. It has also been shown that all-trans

retinoic acid can induce >80% of A-MSCs to undergo epithelial differentiation, as indicated by keratin 18 expression (Brzoska et al., 2005).

1.4.2.3 Umbilical cord-derived MSC (UC-MSC)

Umbilical cord blood cells also harbour multipotent MSCs (Lee et al., 2004). UC-MSCs can be isolated in large numbers from Wharton's jelly, the tissue surrounding the umbilical cord vessels (Mitchell et al., 2003). These cells express similar set of markers to BM-MSCs (Troyer and Weiss, 2008) and can be painlessly isolated and successfully frozen and stored over a long period of time, and hence have the greatest potential for tissue banking (Kenneth and Moise, 2005), despite the high costs.

1.4.2.4 Placenta-derived MSC (P-MSC)

Human placenta has also been shown to contain multipotent progenitors that closely resemble MSCs in their plasticity and markers (Strom and Miki, 2003). P-MSCs have several advantages such as unlimited source of tissue and the non-invasive nature of isolation. However plancental cytotrophoblasts have shown to possess tumour-like properties, thus imposing some safety risks. Furthermore, the actual number and differentiation capacity of P-MSCs from a single placenta may be quite low (Matikainen and Laine, 2005).

It is unlikely that either A-MSCs, UC-MSCs or P-MSCs would be able to generate sufficient quantities of EpSCs for EB treatment. Although, since

they may give rise to type VII collagen-producing fibroblasts, theoretically they could be used to partially and/or temporarily ameliorate severity of the disease.

1.4.3 Haematopoietic Stem Cells (HSCs) for skin regenerative therapy

1.4.3.1 Bone marrow-derived-HSCs (BM-HSCs)

BM-HSCs are among the best characterized adult stem cells. BM-HSCs have been shown to have the plasticity to regenerate various tissues after transplantation (Krause et al., 2001); although the true plasticity of BM-HSCs remains arguable and might be due to a mechanism different from transdifferentiation (Ying et al., 2002). Nevertheless, besides differentiation, there are other potential mechanisms of action of BM-HSCs in injured tissues. These include secretion of various chemokines and cytokines, which stimulate regeneration by inhibiting apoptosis, suppressing immune reactions and increasing angiogenesis, enhancement of proliferation of resident tissue stem/progenitor cells, and cell fusion (Masson et al., 2004, Rabb, 2005, Terada et al., 2002).

1.4.3.1 Umbilical cord blood-derived HSCs (UCB-HSCs)

Umbilical cord blood is more readily available than bone marrow and is better tolerated than bone marrow due to the immunological immaturity of the cells (Eapen et al., 2007). In certain circumstances, cord blood can be

transplanted successfully even if the donor and the recipient are not a perfect match (Wagner et al., 2010b). However, the total number of HSCs is much lower compared to bone marrow (Verneris et al., 2009). Interestingly, when co-cultured with human adult keratinocytes on a fibrin glue/fibroblasts gel, UCB-HSCs can form a regular epithelial sheet consisting of three to four layers of cells (Kamolz et al., 2006).

1.5 CURRENT STEM CELL THERAPY IN RDEB

To date, a number of mouse and human studies have been conducted to evaluate the potential of extra-cutaneous stem cell sources for the treatment of RDEB. It has been shown that embryonic bone marrow transplantation (E-BMT) can ameliorate the pathological abnormalities underlying RDEB (Chino et al., 2008). In this mouse model, bone marrow transfer into the fetal circulation resulted in an increase in deposition of type VII collagen, particularly around developing hair follicles. The authors speculated that E-BMT can potentially be used in utero in the human situation in which prenatal genetic diagnosis indicates *COL7A1* mutations, with predicted severe phenotype. An additional merit of E-BMT is generation of tolerance to the new type VII collagen in a mouse completely lacking type VII collagen because of the inherent *COL7A1* gene mutations. It should be noted, however, that in the above study E-BMT provided bone marrow-derived fibroblasts (BMDFs) to the skin preferentially around hair follicles, and the authors could not observe fully developed, mature anchoring fibrils at the basement membrane. In addition, a “proof-of-principle” study has also

shown that BM-HSC from a wild-type donor improves the basement membrane zone defect in a murine model of RDEB by inducing production of type VII collagen (Tolar et al., 2009). Interestingly, the authors also showed that the infusion of non-haematopoietic bone marrow stem cells failed to extend the life span of *Col7a1*-/- mice and correct murine RDEB. The first clinical trial of allogeneic whole bone marrow transplantation has been reported in 7 RDEB children (Wagner et al., 2010a). Following the infusion of bone marrow or umbilical cord blood, new type VII collagen was noted at the DEJ that continued to increase for at least one year after the bone marrow transplant, and clinical improvement was sustained thereafter in the initial follow up for up to 3 years. Further clinical trials are on-going at this and other centres in the USA (ClinicalTrials.gov identifiers: NCT00478244; NCT01033552; NCT00881556). These trials are looking to refine transplantation protocols, for example in assessing whether intradermal injections of BM-MSCs from the same donor as the transplanted cells might improve clinical response, as well as whether other types of EB might also benefit from bone marrow transplantation. Attempts to use alternative conditioning protocols, such as reduced intensity conditioning, are also being evaluated. Intradermal injection of allogeneic BM-MSCs has been performed in 2 individuals with RDEB (Conget et al., 2010). The injections resulted in increased type VII collagen at the dermal-epidermal junction and improved healing of chronic erosions. The BM correction lasted for ~4 months but there was no sustained clinical benefit beyond this. Nonetheless, these stem cells may partially and/or temporarily

ameliorate the severity of RDEB by providing an alternative source of type VII collagen-producing fibroblasts.

While the bone marrow transplant work highlights the potential of bone marrow cells to correct the intrinsic skin pathology of RDEB, it is clear that much needs to be done in improving the safety and reducing the toxicity of current protocols. Furthermore, elucidating the mechanism involved is essential for improving clinical application of BM-MSCs. Recent experiments in a murine model demonstrate that significant damage to the epidermis due to type VII collagen deficiency and consequent sub-epidermal blistering results in rapid release of HMGB1, which can mobilize a Lin−/PDGFRα+ subset of BM-MSCs into the circulation and accelerate regeneration of the skin by recruiting these cells to the damaged skin and producing BMDKs (bone marrow-derived keratinocytes) and BMDFs in the epidermis and dermis respectively (Tamai et al., 2011). Despite recent findings, the understanding of underlying mechanisms through which the damaged epithelium signals to invoke mobilization and recruitment of BM-MSCs is still limited.

Further clinical trials of intradermal MSCs, from bone marrow or other sources, or sub-populations thereof, are planned in several countries. In addition, it should be remembered that cell therapy using somatic cells can provide clinical benefit for people with RDEB. Use of cultured allogeneic skin fibroblasts by intradermal injection can also lead to improved basement membrane structure and function in RDEB. Indeed, despite the fibroblasts surviving for less than 2 weeks after injection, increased type VII collagen

expression at the DEJ can be seen for several weeks or months (Wong et al., 2008). Another possibility for using somatic cells comes from observations on revertant mosaicism, in which there is a spontaneous correction of the primary genetic defect followed by expansion of the corrected cells (Jonkman et al., 1997). This phenomenon is not rare in EB, perhaps occurring in up to one-third of individuals with non-Herlitz junctional EB (Pasmooij et al., 2007, Jonkman and Pasmooij, 2009, Pasmooij et al., 2005), and it has also been described in RDEB (Almaani et al., 2010, Pasmooij et al., 2010, van den Akker et al., 2012). The possibility of culturing the reverted keratinocytes and then using cultured autologous "naturally corrected" skin grafts has also been investigated (Gostynski et al., 2009) and represents an attractive therapeutic strategy in that it does not rely on genetic manipulation of the cells since the BM genetic defect has been corrected spontaneously.

1.6 PLURIPOTENT STEM CELLS FOR SKIN REGENERATIVE THERAPY

Pluripotent stem cells hold the most promising potential for regenerative cell therapies. One advantage is the capacity of such cells for immortality and self-renewal *ad infinitum*, which could provide an unlimited supply of epidermal cells for skin regeneration. In addition, pluripotent stem cells can also provide a source of other cell types required for skin engineering (Metallo et al., 2008a).

A pluripotent cell line is defined by two indispensible criteria: the ability to self-renew *ad infinitum*, and pluripotency, the ability to differentiate into tissue types from all three extra embryonic germ layers (Figure 4). True pluripotent cells will contribute to all somatic tissues in the fully developed organism, in addition to the germ line of the animal. Due to the combination of these two unique abilities of pluripotent stem cells are considered to be the most attractive cell type for the purpose of regenerative medicine.

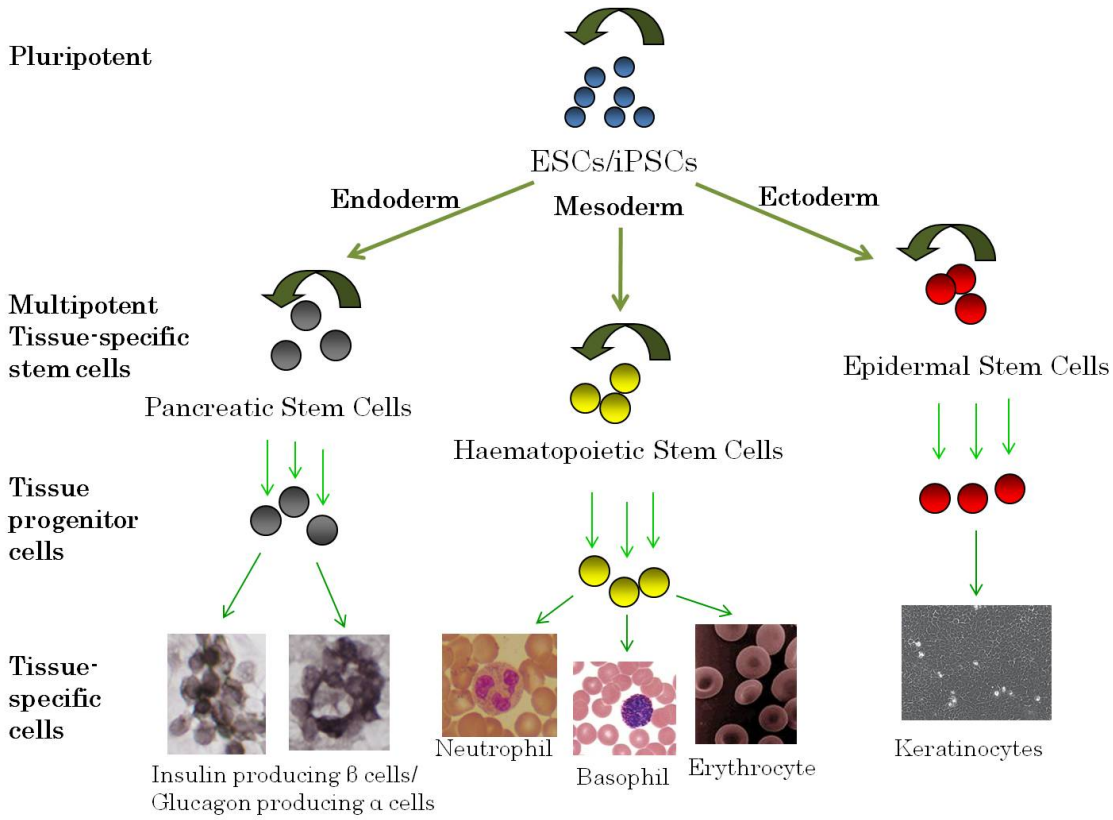

Figure 4 A schematic representation of the differentiation potential of pluripotent cells.
ESCs- embryonic stem cells; iPSCs- induced pluripotent cells. Pluripotent cells are able to self-renew and differentiate towards three germ layers, ectoderm, mesoderm and endoderm, giving rise at first to multipotent tissue-specific stem cells. These multipotent stem cells are also able to self-renew and further differentiate into tissue progenitor cells, which then mature into tissue-specific cells.

1.6.1 Human Embryonic Stem Cells

Human embryonic stem cells (hESCs) were initially isolated from the inner cell mass of preimplantation blastocysts generated by *in vitro* fertilization (Thomson et al., 1998). hESCs can be expanded in culture and forced to undergo directed differentiation to a specified cell type by altering the culture substrate and growth conditions. An extensive collection of studies has shown *in vitro* derivation of somatic cells types of varying degrees of maturity, from hESCs. Differentiation of cells from the ectodermal, endodermal and mesodermal germ layers, has been achieved (Puceat, 2008, D'Amour et al., 2005, Zheng et al., 2006, Green et al., 2003, Levenberg et al., 2002). Derivation of various specialized cell types, for example insulin-producing cells (Segev et al., 2004), has also been accomplished. The biology of hESCs is discussed in detail in Chapter 2.

1.6.2 Induced Pluripotent Stem Cells (iPSCs)

The recent discovery that somatic stem cells can be reprogrammed into a pluripotent state opens new and exciting possibilities for regenerative medicine. This methodology avoids many of the ethical issues associated with the use of hESCs. Reprogramming opened an exciting perspective for generating disease- and patient-specific stem cells. Such cells can potentially provide a unique tool for investigating mechanism in various diseases, drug screening and exploring cell regenerative therapy. Using patient-specific iPSCs in cell regenerative therapy offers the possibility of treatment that does not require immunosuppressive drugs to prevent tissue

rejection, although it remains unclear whether iPSCs will be able to evade an immune response completely.

Initially, iPSCs were successfully derived from adult mouse fibroblasts through the ectopic co-expression of only four genes (Takahashi and Yamanaka, 2006). In this study, the researchers have shown that four factors were sufficient to reprogramme adult fibroblasts into a pluripotent state: Oct3/4, Sox2, Krüppel-like factor 4 (Klf4) and c-Myc. This study initiated extensive research in the field and successful reprogramming quickly progressed to human fibroblasts (Park et al., 2008b, Yu et al., 2007, Takahashi et al., 2007), and other cell types, such as pancreatic ß cells (Stadtfeld et al., 2008a), neural stem cells (Kim et al., 2008, Eminli et al., 2008), mature B cells (Hanna et al., 2008), mouse stomach and liver cells (Aoi et al., 2008), adipose stem cells (Sun et al., 2009) and keratinocytes (Maherali et al., 2008). The reprogramming of dermal fibroblasts was also achieved in the absence of c-Myc (Nakagawa et al., 2008). Other work also demonstrated reprogramming of human fibroblasts into iPSCs using the same factors (Lowry et al., 2008) or Oct4, Sox2, Nanog and Lin28 (Ebert et al., 2009). Primary human fibroblasts have also been reprogrammed with only Oct3/4 and Sox2 and histone deacetylase (HDAC) inhibitor, valproic acid (VPA) (Huangfu et al., 2008b). Intriguingly, the reprogramming of human primary keratinocytes into iPSCs was shown to be at least 100-fold more efficient and twice as quick compared to fibroblasts (Aasen et al., 2008). Moreover, transciptional profiling for stem cell-related genes revealed that keratinocytes are in fact more similar to hESCs than fibroblasts (Aasen et al., 2008). Additionally, unsupervised clustering identified a number of

transcripts expressed in keratinocytes, iPSCs and hESCs but not in fibroblasts. Flow cytometry analysis also revealed expression of putative stem cell markers, such as CD24, in primary keratinocytes but not in fibroblasts. Furthermore, keratinocytes contain higher levels of endogenous Klf4 and c-Myc transcripts compared to fibroblasts which are associated with transcriptional and epigenetic states favourable to reprogramming. Collectively, these intrinsic characteristics of keratinocytes make them a potentially more favourable cell type for reprogramming. Whether keratinocyte-derived iPSCs might also be more amendable to differentiation back towards epidermal cell lineages due to some epigenetic or transcriptional memory, however, is unknown.

The iPSCs were shown to be indistinguishable from ESCs in morphology, proliferation, surface antigens, gene expression, pluripotency and telomerase activity, and were also capable of producing teratomas and germ line-competent chimeras. However, despite significant similarities between iPSCs and hESCs, distinct differences have also been reported. Using DNA methylation mapping, gene-expression profiling and a quantitative differentiation assay, a systematic comparison of 20 established ESC lines and 12 iPSC lines was carried out (Bock et al., 2011). This study produced a 'scorecard' to evaluate the character of both pluripotent cell lines and predict their quality and functional consequences in a high-throughput manner. Despite overall similarities, transcriptional and epigenetic variation is common between pluripotent cell lines and the differences in global gene expression were more pronounced in earlier passages of iPSCs. Concerning the differentiation potential of iPSCs, it has been shown that

some human iPSC lines were less predisposed to differentiate towards haematopoietic, neuroepithelial and neural lineages than hESCs (Miura et al., 2009, Feng et al., 2010, Hu et al., 2010a). While some researchers suggested that this indicates an intrinsically lower capacity of iPSCs for differentiation, an alternative explanation would be that iPSCs retain epigenetic memory of the donor tissue of origin which might influence the differentiation bias of iPSCs (Kim et al., 2010). For example, iPSCs derived from human retinal-pigment epithelial cells are more prone to differentiate back into this cell type than ESCs or iPSCs derived from other tissues (Hu et al., 2010b). The most exhaustive comparison of iPSCs to hESCs revealed loci that can reliably distinguish the two pluripotent cell types (Kim et al., 2010). In addition, the residual iPSC-specific methylation was shown to link these cells to their tissue of origin, which is suggested to affect their differentiation propensity. For example, in iPSCs derived from non-haematopoietic cells, such as fibroblasts and neural progenitors, there can be residual repressive methylation at loci that are required for haematopoietic fates, reducing the blood-forming potential *in vitro* (Kim et al., 2010). However, exogenous supplementation of neural-progenitor-derived iPSCs with the cytokine WNT3A was shown to increase their blood-forming potential, suggesting that incomplete reprogramming owing to epigenetic marks can be surpassed by manipulating culture conditions (Kim et al., 2010). These findings show that the 'epigenetic memory' of the donor cell can be exploited in order to manipulate iPSCs differentiation fate.

In recent years, a variety of reprogramming methods have been investigated. With regards to clinical translation, the use of viral vectors for

reprogramming poses several problems and safety concerns, such as insertional mutagenesis and difficulty in storage and quality control. Therefore, several non-viral systems have been explored in inducing reprogramming. These include transposon systems (Soldner et al., 2009, Woltjen et al., 2009) and use of a single multiprotein expression vector (Kaji et al., 2009). It has also been shown that reprogramming can be achieved with the use of cell-penetrating recombinant proteins (Zhou et al., 2009). More recently a single transfer of ESC-derived proteins into primary cultured adult mouse fibroblasts has been shown to achieve full reprogramming to the pluripotent state (Cho et al., 2010). The non-viral systems are discussed in more detail in Chapter 3.

Despite significant advances in the field of iPSC research, crucial criteria have to be met before clinical application of these cells can be accomplished. Firstly, an efficient protocol without the use of any potentially harmful components needs to be developed. Secondly, stringent standards for iPSCs characterization and analysis have to be devised. Understanding the exact genetic and epigenetic events that occur during reprogramming will be crucial for designing experimental approaches. And finally, refined differentiation protocols are needed to obtain the desired cell type that will enable researchers to explore the full potential of iPSCs.

1.7.3 Therapeutic potential of pluripotent stem cells for skin regeneration depends on understanding epidermal morphogenesis

Although very much at a pre-clinical level, pluripotent stem cell sources such as hESCs and iPSCs offer new opportunities for the development of clinically relevant therapies. Disease-specific iPSC lines could be derived to study mechanisms at a molecular level and serve as a disease model for therapeutic drug discovery (Park et al., 2008a). With respect to RDEB, iPSC lines could be used to study developmental and signalling pathways underlying blister formation as well as the predisposition of people with RDEB to develop squamous cell carcinomas. In addition, genetically-corrected iPSCs could be used to generate disease-free skin cell-progenitors with potential application in autologous cell therapy, and protocols to obtain genetically-corrected iPSCs from dermal fibroblasts and epidermal keratinocytes have been reported (Raya et al., 2010). Moreover, the phenomenon of revertant mosaicism could also be combined with iPSC technology, with the added advantage of not having to correct the BM gene abnormality (Pasmooij et al., 2010). The iPSC approach means that different cell types to restore type VII collagen could be created to be used in conjunction, such as bone marrow cells for systemic delivery and keratinocytes or fibroblasts for local treatment. However, all these goals can only be achieved if an efficient differentiation protocol that would direct pluripotent stem cells towards epidermal lineages is developed.

The main prerequisite that has to be met when manipulating cell fate of a pluripotent cell *in vitro*, is a thorough understanding of the embryonic

morphogenesis of a given tissue, in order to achieve efficient production of desired cell type. For example, a three-stage process for the efficient and reproducible differentiation of hESCs to hepatocytes was achieved by recapitulating liver development *in vivo* (Hay et al., 2008). In this approach hESCs were first primed towards definitive endoderm with activin A and sodium butyrate, then further differentiated to hepatocytes with dimethyl sulphoxide and matured hepatocyte growth factor and oncostatin M. Therefore, in order to develop an efficient differentiation protocol for derivation of skin cell lineages from hESCs, epidermal morphogenesis *in vivo* must be understood.

1.8 EPIDERMAL MORPHOGENESIS

A number of articles have thoroughly reviewed epidermal morphogenesis and homeostasis (Fuchs and Raghavan, 2002, Simpson et al., 2011, Blanpain and Fuchs, 2009).

1.8.1 Early ectodermal development and the "default model"

After gastrulation, the embryo surface emerges as a single layer of neuroectoderm, which will ultimately specify the nervous system and skin epithelium. This decision is guided by Wnt signalling, which blocks the ability of ectoderm to respond to fibroblast growth factors (FGFs) (Figure 5). In the absence of FGF signalling the cells express bone morphogenetic proteins (BMPs), and become fated to develop into epidermis. Contrarily, neural fate arises when, in the absence of a Wnt signal, the ectoderm is able

to receive and translate activating cues by FGFs, which then attenuate BMP signalling through inhibitory cues (Chang and Hemmati-Brivanlou, 1998, Stern, 2005). This "default model" of neural differentiation has been widely accepted. Two hallmark studies revealed that depletion of three different BMPs (2, 4 and 7), together with the related protein anti-dorsalising morphogenetic factor (ADMP) or with abrogation of the organizer region where ADMP is normally expressed, results in the formation of a massive brain (Reversade et al., 2005, Reversade and De Robertis, 2005). These results are taken to support the "default model", indicating that inhibition of BMP activities in the early embryo is sufficient to cause great expansion of the nervous system at the expense of epidermis.

The embryonic epidermis that results from this process consists of a single layer of multipotent epithelial cells. It is covered by a transient protective layer of tightly connected squamous endodermis-like cells, known as a periderm, which are shed once the epidermis has stratified and differentiated (Akiyama et al., 1999, Polakowska et al., 1994). This single-layerd epidermis persists from mouse embryonic day 9.5 (E9.5) to E12.5. As mesenchymal cells populate the skin, the signals are transmitted that instruct the stratification of the epidermis and direct the positioning of down growths that denote the initiation of hair follicle morphogenesis (Koster and Roop, 2007).

Simultaneously, the innermost, basal, layer of the stratifying epidermis produces numerous extracellular matrix (ECM) proteins and growth factors, which are organized into an underlying BM. The BM does not only serve as

a growth-supporting platform, but also as a physical border between the epidermis and the dermis.

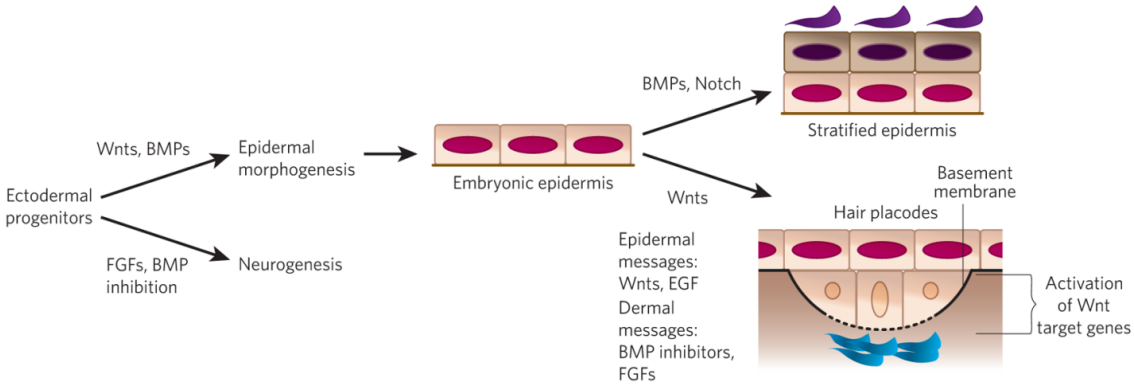

Figure 5 Early signalling during embryonic epidermogenesis.
In the absence of Wnt signalling, ectodermal progenitors respond to FGFs, downregulate BMP signalling and progress towards neurogenesis. Wnt signalling blocks the ability of early ectodermal progenitor cells to respond to FGFs, allowing them to respond to BMP signalling and adopt an epidermal fate forming single-layered embryonic epidermis. The cells that fail to respond to Wnts become fated to become epidermal cells through BMP, EGF and Notch signalling. The cells that do respond to Wnt signalling also receive underlying FGF and BMP inhibitory signals from the mesenchyme. These cumulative signals instruct the cells to produce the hair placode. From (Fuchs, 2007).

1.8.2 Stratification

Epidermal stratification is brought about by specific transcription factors (Mack et al., 2005). The initial stages of stratification (E12.5–E15.5) are marked by suprabasal cell division, which is aimed at rapid expansion of epidermal cells. However, these suprabasal cells soon undergo complete differentiation. Stratification is generally completed by E17.5, by which point the epidermis consists of an inner layer of highly proliferative basal cells and layers of terminally differentiating, suprabasal cells.

During transit to the skin surface, keratinocytes undergo a dramatic transformation (Fuchs and Raghavan, 2002) . The first paper to show that

basal cells in the mammalian epidermis undergo asymmetric divisions to drive stratification during epidermal morphogenesis and identified the cytoarchitectural elements regulating this process suggested that this process occurs through reorientation of mitotic spindles in the basal layer (Lechler and Fuchs, 2005). While basal cells remain attached to an underlying matrix and proliferate, some of their daughter keratinocytes enter the spinous layer, "stratum spinosum", through asymmetric mitoses, where they exit the cell cycle, grow larger and establish robust intercellular connections. These suprabasal cells no longer have cell–matrix adhesions, which completely alters their cell polarity and cytoskeletal architecture (Watt, 2002). Cells in the granular layer, "stratum granulosum", flatten and assemble a water-impermeable cornified envelope underlying the plasma membrane. Finally, corneal layer, "stratum corneum", keratinocytes release lysosomal enzymes to degrade major organelles, become completely squamous and are tightly crosslinked together to complete the cutaneous barrier (Michel et al., 1988, Candi et al., 2005, Kalinin et al., 2002).

1.8.3 Keratin expression

In epidermal keratinocytes, intermediate filaments fill the cytoplasm and mechanically connect hemidesmosomes at the basement membrane and desmosomes at the lateral membrane to the cell nucleus. The intermediate-filament-associated protein plectin binds α6β4 integrin at hemidesmosomes but also associates with nesprin 3, a protein embedded in the outer nuclear membrane (Wilhelmsen et al., 2005). At the same time, its homologue,

nesprin 2, regulates nuclear envelope morphology (Luke et al., 2008). Therefore, in keratinocytes the desmosome–nesprin complex creates a unique mechano-sensory mechanism, which responds to mechanical forces at the periphery of the cell by directly influencing nuclear activity during epidermal morphogenesis.

Well-defined changes in keratin gene expression accompany epidermal morphogenesis and stratification (Fuchs and Green, 1980). Keratin 8 and its binding partner keratin 18 are expressed earliest during embryonic development (Oshima et al., 1983). Keratins 8/18 serve as a marker for simple epithelia. Keratin 19 can associate with different partners and is expressed in embryonic, simple, and stratified epithelia (Stasiak and Lane, 1987). Keratins 5/14 are markers for the basal proliferative layer of epidermis. As cells enter the spinous layer, they switch off the expression of genes encoding keratins 5 and 14. Simultaneously, spinous cells switch on the expression of genes encoding keratins 1 and 10 to form an even more robust intermediate filament network that provides resistance against mechanical stresses at the body surface. Cornification involves biochemical crosslinking of various keratinocyte proteins such as loricrin and involucrin by transglutaminases (TGases), but it eventually terminates with nuclei and organelles being broken down by intracellular and secreted proteases, including a specialized caspase (Eckhart et al., 2000) Granular cells express additional structural proteins that are deposited beneath the plasma membrane. These proteins become enzymatically cross-linked, forming an indestructible sac, which acts as a water-impermeable barrier (Candi et al., 2005). It serves as a scaffold for specialized lipid bilayers that are extruded

from intracellular lamellar granules into the extracellular space between dead flattened stratum corneum cells, known as squames. The squames are interposed by lipids on the outside and filled with a fibrous mass of keratins that is encased by the cornified envelope (Fuchs and Raghavan, 2002, Reversade et al., 2005). The cellular junctions in stratum corneum are called corneodesmosomes. Those contain the cadherins desmoglein 1 (DSG1) and desmocollin 1 (DSC1), which must be cleaved by serine proteases to allow proper shedding of the outer epidermis (Caubet et al., 2004).

Therefore mature epidermis displays tissue-level polarization with asymmetric distribution of signalling activity, protein expression and cytoarchitectural organization that reflects the unique functions of its multiple layers.

1.8.4 Integrins

Keratinocytes of the basal layer are inherently polarized as their lower surface is anchored to the basement membrane by integrins (Figure 6). Integrins are heterodimeric transmembrane receptors that bind specific ECM components, such as laminin, collagens and fibronectin via their extracellular domains (Watt, 2002). Each integrin is a heterdimer of two glycoproteins, known as α- and ß-subunits, that are non-covalently associated. As many as 11 integrin heterodimers have been described in epidermal keratinocytes (Margadant et al., 2010). The most abundant integrins in the epidermis are α2ß1 (collagen receptor), α3ß1

(predominantly, laminin-332 and -511/521 receptor) and α6β4 (mainly laminin-332 receptor) (Watt and Hertle, 1994, Margadant et al., 2010).

In normal, undamaged epidermis, integrin expression is confined to the basal layer and outer root sheath of the hair follicle. α6β4 integrin has a special role in organizing large integrin complexes called hemidesmosomes (Tsuruta et al., 2003). Hemidesmosomes connect to the BM through α6β4 integrin and the transmembrane protein bullous pemphigoid antigen 2 (BPAg2; also known as type XVII collagen), and are tethered to intermediate filaments by the linker proteins plectin and bullous pemphigoid antigen 1e (BPAg1e; also known as BP230 or dystonin). Clusters of β1 integrins are interspersed with hemidesmosomes on the basal surface of basal keratinocytes, but the majority of β1ntegrins are found to form an "O" ring at the periphery of the cell (Jensen et al., 1999)

Integrin heterodimers regulate cell growth and differentiation through signal transduction (Hynes, 1992, Dedhar and Hannigan, 1996). Transmembrane connection is achieved through association of integrin tails with actin via adaptor proteins on the intracellular side and recruitment of various factors to their cytoplasmic tails. For example, through such signal transduction α3β1 integrin plays a crucial role in morphogenesis of the BM through influencing the ability of keratinocytes to secrete ECM components (McMillan et al., 2003). Total loss of α3 integrin results in neonatal lethality and sub-epidermal blistering due to a discontinuous BM between hemidesmosomes (DiPersio et al., 1997). Epidermal-specific deletion of α3

integrin results in duplicated areas of the BM and microblisters at the DEJ (Margadant et al., 2009).

Initial *in vitro* experiments showed that terminal differentiation and cell cycle withdrawal of cultured keratinocytes is induced by their release from an underlying matrix (Green, 1977, Watt, 1994). Furthermore, in intact epidermis, keratinocytes cease proliferating as they leave the basal layer, which coincides with loss of contact with the BM and suppression of integrin expression. This suggests a vital role for integrins in epidermal homeostasis. It has been hypothesized that stratification-associated down-regulation of integrins diminishes proliferation in the suprabasal epidermal layers (Margadant et al., 2010, Watt, 2002). Under normal circumstances, mitogenic signalling from epidermal growth factor receptor (EGFR) via the mitogen-activated protein kinase (MAPK) pathway is restricted to basal keratinocytes, which have high expression of various integrins (De Potter et al., 2001, Aplin and Juliano, 1999). Integrins can bind to receptor tyrosine kinases, including EGFR, providing a possible mechanism for how they may regulate proliferation (Muller et al., 2008, Guo and Giancotti, 2004).

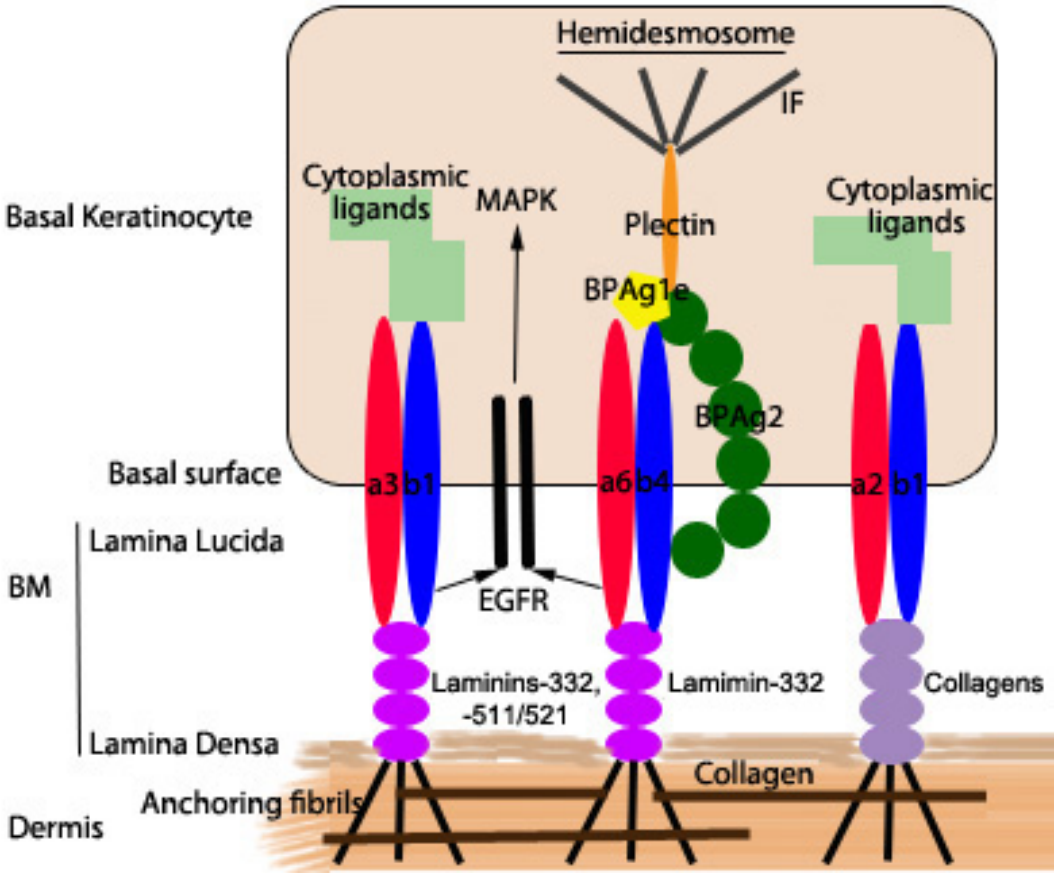

Figure 6 Constitutively expressed integrins of the basal epidermal layer. Basal keratinocytes are attached to the BM (composed of lamina lucida and lamina densa) via numerous hemidesmosomes and integrin-based adhesions. Hemidesmosomes connect to the BM through α6ß4 integrins and the transmembrane protein BPAg2 (also known as type XVII collagen), and are tethered to intermediate filaments (IF) by plectin and BPAg1e proteins. α3ß1 and α2ß1 integrins are receptors for laminins-332, -511/521 and collagens, respectively. Through association of integrins with ECM proteins basal epidermal layer is attached to underlying dermis via anchoing fibrils and collagen. Both α3ß1 and α2ß1 integrins provide transmembrane connections by recruiting several cytoplasmic ligands to their intacellular domains.

Defects in integrin expression have a profound effect on all aspects of skin function. In mice, deletion of ß4 integrin prohibits formation of hemidesmosomes, resulting in severe blistering (Dowling et al., 1996, van der Neut et al., 1996). Loss of ß4 integrin also appeared to induce apoptosis of basal cells (Dowling et al., 1996), whereas deleting the cytoplasmic tail of ß4 impairs proliferation (Murgia et al., 1998). On the other hand, deletion of α6 integrin, the partner of ß4 in hemidesmosomes, increases proliferation

and skin inflammation (Niculescu et al., 2011). In humans, defective expression of the α6ß4 heterodimer has been implicated in JEB and associated with congenital gastric outlet obstruction, pyloric atresia (Gil et al., 1994, Niessen et al., 1996). Furthermore, in squamous cell carcinoma overexpression of α6ß4 integrin is seen throughout the tumour mass and correlates with poor prognosis (Downer et al., 1993, Van Waes et al., 1995). Consistently, in hyperproliferative human conditions, such as psoriasis and carcinomas, integrin expression is seen beyond the basal layer (Hertle et al., 1992, Guo and Giancotti, 2004, Haase et al., 2001). Conversely, deletion of ß1 integrin in skin produces a hypoproliferative epidermis with more differentiating keratinocytes than in normal epidermis (Raghavan et al., 2000, Brakebusch et al., 2000). However, this also results in extensive epidermal blistering. Therefore it remains difficult to dissect the potential effects of faulty integrin expression on skin homesostasis and integrity.

Importantly, a range of human disorders, such as junctional EB, EB simplex and bullous pemphigoid, result from integrin-based adhesion being compromised by mutation or by autoimmune responses and are characterized by varying degrees of epidermal fragility and blistering (McGrath et al., 1995, Groves et al., 2010, Gache et al., 1996, Gil et al., 1994).

1.8.5 Epidermal cell junctions

Cadherin-based junctions, including adherens junctions and desmosomes, are abundant in basal keratinocytes. Epidermal adherens junctions are

composed of the classic cadherins such as E-cadherin and placental cadherin (P-cadherin; also known as cadherin 3). Variations in different cadherin expression play an important role in epidermal morphogenesis because they allow keratinocytes to be sorted into discrete epidermal layers (Gumbiner, 2005, Runswick et al., 2001). Gap junctions in the epidermis allow direct cytoplasmic communication between keratinocytes and are implicated in epidermal morphogenesis and disease (Lai-Cheong et al., 2007, Mese et al., 2007).

In basal keratinocytes, cellular junctions have a polarized organization and contribute to mitotic spindle orientation during stratification. In undifferentiated basal keratinocytes actin filaments are highly associated with cell–matrix junctions, whereas during differentiation they become coupled to intercellular junctions (Perez-Moreno et al., 2003, Vaezi et al., 2002, Kubler et al., 1991). *In vitro*, cadherin–catenin complexes at adherens junctions are linked into a cortical ring of bundled microfilaments, which effectively couples the actin cytoskeletons of neighbouring cells (Vasioukhin et al., 2000). It is believed that these actin-linked adhesions bind motor proteins such as myosin to generate the tension required for a keratinocyte to move over a neighbouring cell (Perez-Moreno et al., 2003, Vaezi et al., 2002).

Stratification induces a marked increase in the amount of desmosomal cellular junctions. Desmosomes are built from clustered transmembrane cadherins called desmogleins (DSGs) and desmocollins (DSCs). These bind to plakoglobin and plakophilins, which are members of the intracellular

armadillo protein family. In turn, this binding recruits the cytolinker desmoplakin, which binds keratin intermediate filaments (Hatsell and Cowin, 2001, Green and Simpson, 2007). Through this chain of interactions, desmosomal components directly link intercellular junctions and the intermediate filament cytoskeleton. Changes in desmosomal cadherin expression modulate intracellular signalling and control epidermal differentiation (Merritt et al., 2002, Hardman et al., 2005, Elias et al., 2001, Kljuic et al., 2003). It has been suggested that desmosomal cadherins might promote the switch from proliferation to differentiation upon stratification (Lechler and Fuchs, 2007, Teuliere et al., 2004, Green and Simpson, 2007).

In the granular layers, tight junctions help to promote an environmental barrier for the skin (Niessen, 2007). Tight junctions seal multicellular sheets by forming a belt-like adhesion between cells, allowing the passage of only small molecules and ions (Morita and Miyachi, 2003). Numerous proteins are present in the tight junctions, including claudins, junctional adhesion molecules, occludins and tricellulin, as well as scaffolding proteins of the zonula occludens family (Niessen, 2007). Tight junctions play a crucial role in the skin immune response by allowing antigen sampling by resident Langerhans cells (Kubo et al., 2009).

1.9 APPROACHES FOR EPIDERMAL DIFFERENTIATION

To date there have been numerous attempts to devise an efficient protocol for differentiation of hESCs towards keratinocyte cell lineages. Epidermal differentiation has been reported in murine (Bagutti et al., 2001, Kawasaki

et al., 2000, Coraux et al., 2003a) and human ESCs (Aberdam et al., 2008, Gambaro et al., 2006, Ji et al., 2006, Metallo et al., 2010c, Schuldiner et al., 2000, Troy and Turksen, 2006). Keratinocyte differentiation in embryoid bodies has been spontaneous (Bagutti et al., 2001, Bagutti et al., 1996) or induced with type IV collagen (Ji et al., 2006). Alternative approaches included co-culture (Troy and Turksen, 2006) and through the cultivation of nodules derived from teratomas (Green et al., 2003), as well as direct culture as monolayer (Coraux et al., 2003a). However, in all of the above protocols the commitment was inefficient, resulting in a highly heterogeneous cell population along with remaining undifferentiated ES, which could still produce teratomas. Furthermore, these hESC derivatives exhibited a lower proliferative capacity compared with primary keratinocytes, and immortalization was required to extend the lifespan of these cultures (Iuchi et al., 2006).

The current understanding is that BMP-4 and all-trans-retinoic acid (ATRA) act synergistically to achieve hESCs differentiation to keratinocytes with BMP-4 acting to block neural differentiation and ATRA directing cells to an epithelial fate (presumably through induction of ΔNp63 isoform expression). It has been demonstrated that the exogenous expression of ΔNp63 isoform specifically is required for epidermal commitment of murine keratin 18-positive ectodermal cells into keratin 14-positive keratinocytes (Aberdam et al., 2007). Further investigation revealed that ΔNp63 isoform is expressed soon after BMP-4 treatment, along with the simultaneous expression of direct or indirect p63-target genes associated with epidermal morphogenesis (Medawar et al., 2008).

Retinoids are potent regulators of cell proliferation and differentiation and are highly involved in embryonic development. In skin retinoids have profound effects. Topical administration of ATRA to photoaged skin reduces wrinkling (Kligman et al., 1986, Weiss et al., 1988). This effect is brought by thickening and compaction of the stratum corneum and increased keratinocyte proliferation. On the other hand, systemic retinoid treatment in psoriasis ameliorates the lesions by an opposing mechanism of decreasing keratinocyte poroliferation (Williams and Elias, 1981, Orfanos and Runne, 1978). In culture, ATRA inhibits growth of the rapidly proliferating keratinocytes, while stimulating growth of quiescent cells (Varani et al., 1989). In addition, ATRA may inhibit the *in vitro* production of ECM proteins by keratinocytes, and therefore reduces adhesion. Interestingly, the concentration required for matrix production inhibition is much higher than that required to stimulate proliferation. This might in turn explain the different effects in topical and systemic administration of retinoids. ATRA has been shown to inhibit the terminal differentiation of keratinocytes *in vitro* by modulating p63 isoform expression (Bamberger et al., 2002) and is a caudalizing factor that directs ESC-derived neuroepithelia to become motor neurons (Li et al., 2005, Wichterle et al., 2002). Interestingly a study that used ATRA to induce formation of posterior neuroepithelia also reported that early ATRA application induced unidentified non-neural cell fates (Pankratz et al., 2007).

ATRA mediates cellular responses by binding to nuclear retinoic acid receptors (RARs), which in turn modulate transcription through several pathways. Within 48 hours of ATRA administration, undifferentiated

hESCs, which express RARα, initiate p63 transcription, predominantly the ΔNp63 isoforms (Schuldiner et al., 2000). On the other hand, mice expressing dominant negative forms of RARα under the *K14* promoter show reduced p63 expression and severe defects similar to those observed in *p63* knockouts (Chen and Lohnes, 2005, Saitou et al., 1995). Furthermore, RA has been shown to induce ΔNp63 expression in RAR αγ-null keratinocytes in the presence of elevated RARα levels (but not RARγ) (Chen and Lohnes, 2005). That means that RA may regulate p63 transcription, directly or indirectly, in hESCs because of differential expression of RAR subtypes. It has been shown that ectodermal derivatives of hESCs can acquire epithelial fates in response to ATRA in a stage-specific and dose-dependent manner (Metallo et al., 2008b). These authors have showed that significant keratinocyte induction occurred only when embryoid bodies were cultured with 1μM ATRA at the early stages of differentiation. This process is marked by significant increases in keratin 18 and p63 transcription and relative decreases in pluripotency (e.g. Oct-3/4, SSEA-4, Nanog) and neural gene transcription. After early induction with ATRA and BMP-4, these keratin 18+/p63+ cells are subcultured in a defined epithelial medium and ultimately give rise to keratin 14+/p63+ keratinocytes.

Functionality of these differentiated keratinocytes was assessed by their ability to undergo stratification and cornification at the air–liquid interface induced stratification (Metallo et al., 2010a). This was confirmed by immunofluorescence microscopy for terminal differentiation markers, including keratin 10, involucrin, and filaggrin. However, the overall tissue morphology was significantly different compared with human skin samples.

Further characterization of keratinocytes obtained by the method described above revealed that although transcriptional patterns were similar to those in primary keratinocyte cultures, genes associated with signal transduction and ECM were upregulated (Metallo et al., 2010a). Elevated signalling along these pathways might ultimately interfere with the terminal differentiation program of keratinocytes and explain the results obtained in organotypic culture experiments. Finally elevated expression of embryonic genes such as *CPN6* and *HAND2* was also observed, indicating the presence of contaminating embryonic cell types.

A significant advantage of ATRA-induced differentiation is that can be efficiently performed in defined medium as opposed to feeder systems (Aberdam et al., 2008) and/or undefined medium application (Ahmad et al., 2007, Coraux et al., 2003a). Chemically defined serum-free media rather than media containing serum was chosen to avoid serum that is subject to batch variations and potential contamination by various pathogens that could be transmitted in animal serum (Merten, 1999, Froud, 1999).

1.10 SUMMARY

The epidermis is a thin layer of stratified squamous epithelium that rests on top of a BM, which separates it and its appendages from the underlying mesenchymally-derived dermis. The epidermis performs vital functions in protecting the organism from physical and environmental stress as well as providing the first defense against pathogens. Despite its remarkable stability, the epidermis is a highly dynamic tissue which undergoes constant

turnover and regeneration. This epidermal homeostasis is supported by adult stem cells within the skin.

However, in some genetic skin conditions, such as EB, skin regeneration is severely impaired. The absence of one of the critical components within the DEJ leads epidermis to detach from the underlying dermis causing constant blistering. Currently, there is no curative treatment available for patients with EB. It is very likely that the persistent blisters might diminish the endogenous stem cell pool and/or disrupt epidermal stem cell niches. Therefore, a potential cell regenerative therapy should be aimed at restoring the pool of endogenous epidermal progenitors.

Several stem cell types have been proposed and evaluated, including adult BM-MSCs and HSCs. However, pluripotent stem cells hold the most promise in regenerative cell therapy due to their unique capacities for infinite self-renewal and differentiation potential. Therefore, the overall aim of thesis was to investigate various differentiation strategies to obtain highly proliferative epidermal progenitors from human embryonic stem cells.

1.11 PROJECT AIMS AND HYPOTHESIS

Data presented in this study can be divided into two working hypotheses:

1.11.1 Hypothesis 1

The conditions for culture and/or derivation of pluripotent stem cells can be improved towards clinical grade for subsequent derivation of epidermal progenitors.

General aims:

1. To develop conditions for pluripotent stem cell culture and/or derivation that would enable potential therapeutic application of these cells.

2. To confirm the pluripotency potential of stem cells cultued and/or derived under such conditions.

Chapters 2 and 3 focus on this hypothesis. Chapter 2 describes the adaptation of the KCL-OO2 hESC line to defined feeder-free conditions, and Chapter 3 describes the protocols for non-viral derivation of iPSCs. The specific hypotheses and aims are described for each set of experiments separately.

1.11.2 Hypothesis 2

Recapitulating embryonic epidermal morphogenesis, in particular the inductive effects of mesenchyme, *in vitro* is key to efficient production of epidermal progenitors from hESCs.

General aims:

1. To test different methods for inducing epidermal differentiation of pluripotent stem cells.

2. To evaluate the efficiency of the protocol based on epidermal marker expression.

Chapters 4-6 relate to this general hypothesis, and specific hypotheses and aims are outlined for each set of experiments separately.

CHAPTER 2 HUMAN EMBRYONIC STEM CELLS: ADAPTATION FOR FEEDER-FREE CULTURE AND VALIDATION OF PLURIPOTENCY

HYPOTHESIS

Human ESC line KCL-002 could be successfully adapted to growth in feeder-free culture conditions, while still maintaining all the properties of an ESC line, such as expression of marker genes and proteins associated with hESCs, spontaneous differentiation through embryoid body formation and form teratomas, containing all three germ layers *in vivo*.

AIMS

1. Adapt KCL-002 hESC line to growth under feeder-free culture conditions
2. Examine levels of expression of the pluripotency-associated marker genes Nanog and Sox-2 in the KCL-002 hESC line
3. Investigate expression of the pluripotency-associated proteins in KCL-002 hESC line
4. Confirm the pluripotency of the KCL-002 hESC line *in vitro* through embryoid body formation

5. Determine the pluripotency of the KCL-002 hESC line *in vivo* by performing a teratoma assay.

2.1 INTRODUCTION

2.1.1 Definition of ESC

An ESC line is defined by two indispensible criteria: the ability to self-renew *ad infinitum*, and pluripotency, the ability to differentiate into tissue types from all three extra embryonic germ layers. True ESCs will contribute to all somatic tissues in the fully developed organism, in addition to the germ line of the animal. Due to the combination of these two unique abilities, ESCs are considered to be the most attractive cell type for the purpose of regenerative medicine.

2.1.2 Derivation of hESC lines

For the derivation hESC lines, embryos between 5 and 7 days post-fertilisation, termed blastocysts, are used. A blastocyst is a fluid-filled cavity, termed blastocoele, which contains a cluster of cells called the inner cell mass (ICM) at its apical side and a thin outer trophoblast layer, or trophectoderm (TE), enclosing the cavity. During normal embryogenesis, the TE, becomes the placenta which supports the development of the ICM into a foetus. So it is the ICM that harbours those unique pluripotent cells that have the potential to develop into any tissue of the body. In the early 1980s, Evans and Kaufman (Evans and Kaufman, 1981) and Martin (Martin, 1981) independently identified the promise that lies within the isolation and propagation of the ICM. The mouse ICM was successfully isolated and propagated in an embryonic state under specific culture

conditions, generating the first ESC lines. When injected into a developing mouse blastocyst, these ESCs were shown to create chimeric mice contributing to all cell lineages including the germ lines, thus proving their capacity for pluripotent differentiation (Bradley et al., 1984).

In 1998, the first hESC line was derived and characterised (Thomson et al., 1998), and since then numerous lines have been established. Human ESC lines can be broadly classed into two groups- normal and specific-mutation carrying lines, for example, mutations linked to cystic fibrosis (Pickering et al., 2005) or Huntington disease (Stephenson and Braude, 2010). The hESC lines carrying a mutation for a specific genetic disease can be used to investigate the onset and progression of genetic disorders and facilitate drug development which could otherwise not be performed in human patients. These specific-mutation carrying lines are derived from clinically unsuitable embryos following pre-implantation genetic diagnosis (PGD) (Pickering et al., 2003). When PGD demonstrates positivity for alleles carrying a risk of a certain genetic conditions, embryos are often donated for medical research. In addition the blastocysts used for hESC purposes may be donated to medical research by couples who undergone *in vitro* fertilisation (IVF) (Reubinoff et al., 2000, Lanzendorf et al., 2001, Amit and Itskovitz-Eldor, 2002, Cowan et al., 2004). IVF generally produces a large number of embryos, which is supernumerary to reproductive desire requirements. Poor-quality embryos which would otherwise be discarded, provide an alternative source (Mitalipova et al., 2003).

The derivation of hESCs at King's College London is under license from the UK Human Fertilisation and Embryology Authority (HFEA; research

license number R0133) and also has local ethical approval (UK National Health Service Research Ethics Committee Reference 06/Q0702/90). In accordance with HFEA regulations, a sample of each line that is derived is deposited in the UK Stem Cell Bank for distribution to academic and research centers internationally. Figure 7 shows the laser-assisted process of hESC line derivation, as currently used at King's College London.

Alternatively hESC lines could be derived without the destruction of embryos. This technique involves a single cell biopsy of an 8-cell embryo, and the removed cell (a blastomere) can in some instances be cultured to produce a stable hESC line (Klimanskaya et al., 2006, Chung et al., 2006). The biopsy is not detrimental to the development of the embryo, as the cell is quickly replaced by division of a neighbouring blastomere.

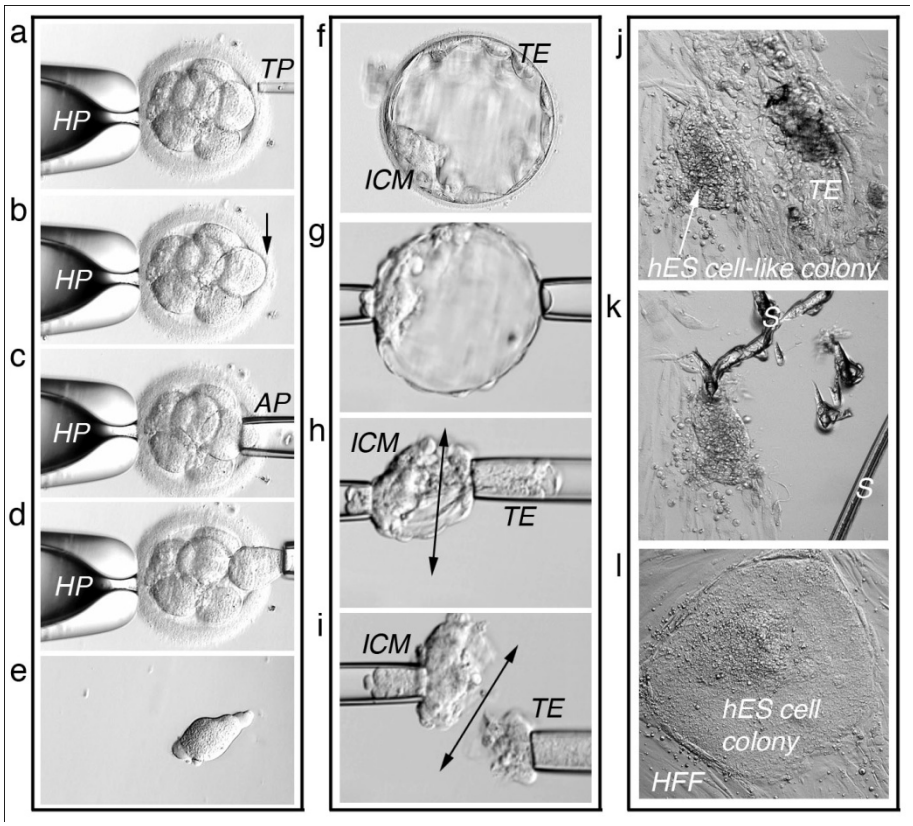

Figure 7 Laser-assisted derivation of hESCs.
(a-e) Single blastomere biopsy from a cleavage-stage (day-3 post-fertilization) embryo. **(a)** Cleavage-stage embryo held with a holding pipette (HP) is exposed to a gentle stream of acid Tyrode's solution coming through a Tyrode's pipette (TP); **(b)** A blastomere popping through a Tyrode's solution-drilled hole in the zona pellucida (arrow); **(c, d)** The blastomere is carefully aspirated using an aspiration pipette (AP); **(e)** The biopsied blastomere is examined for the presence of a nucleus and DNA is used for mutation diagnosis; **(f–i)** Laser-mediated ICM dissection. The ICM and TE of a well-developed blastocyst are separated with a series of laser pulses (double-arrow); **(j, k)** ICM outgrowth on mitotically inactivated human foreskin fibroblasts (HFFs), 5–7 days after plating; **(j)** Two distinct cell populations within an outgrowth; hESC-like colony and TE cells could be distinguished under higher magnification about 5–7 days post-plating; **(k)** TE is dissected out with a needle. S, needle scratches on tissues culture plastic; **(l)** Typical 12–15-day old hESC colony on mitotically inactivated HFF. Figure taken from (Ilic et al., 2012)

2.1.3 Germ layer differentiation in human embryogenesis

Pluripotency of hESC lines presumes that *in vitro* differentiation of these cells would recapitulate embryogenesis in the womb and lead to formation of all three primitive germ layers, the ectoderm, endoderm and mesoderm. Germ layers are defined as cellular layers into which embryos differentiate

in the gastrula stage and from which the organs and tissues of the body develop through further differentiation.

All three embryonic germ layers in humans are well characterised with regard to their development during embryogenesis. An appreciation of the germ layers is crucial in order to design a system of tissue culture techniques that encourage formation of cell types from a particular lineage.

During the blastocyst stage of human embryonic development, the primitive endoderm forms, as an outer layer of the ICM, and will eventually form only the extraembryonic support structures such as the yolk sac. Implantation and penetration of the blastocyst into the endometrium lining of the uterus occurs, followed by primitive ectoderm formation. Cavitation of the inner most group of cells in the primitive ectoderm generates a two-layered embryonic structure termed the epiblast. It is from this structure, appearing around 16 days post-fertilisation that gastrulation and organogenesis occur. Dynamic cell migrations and focal gradients of numerous chemical mediators orchestrate the appearance of a body plan.

2.1.4 The embryonic stem cell control network

The concept of 'stemness' was first developed following expression profile studies comparing gene expression in human embryonic stem cells to both mouse ESC and cancer cell lines (Ramalho-Santos et al., 2002, Sato et al., 2003, Sperger et al., 2003, Richards et al., 2004, Zeng et al., 2004b, Wei et al., 2005) to describe a portfolio of commonly expressed genes whose combined functional proteins define a cell with stem-like attributes. Despite

considerable similarities in gene expression between different hESC lines, each line has also been proposed to have its own expression signature (Abeyta et al., 2004, Skottman et al., 2005). Nevertheless, a core subset of genes associated with hESC pluripotency has been identified.

2.1.4.1 Octamer-3/4 and Sox-2

The first pluripotency-associated transcription factor to be identified, Oct-3/4 (Octamer-3/4) is a homeodomain-containing POU-family transcription factor protein, encoded by the *Pou5f1* gene. The POU family of transcription factors will bind to the octamer nucleotide sequence ATTTGCAT, in the promoter regions of numerous genes and functional activity of Oct-3/4 in pluripotent cells has been shown (Okamoto et al., 1990, Rosner et al., 1990, Scholer et al., 1990). Knockdown models of Oct-3/4 using RNA interference were correlated with loss of ESC phenotype and morphology *in vitro* (Matin et al., 2004), and regulation of the levels of the active Oct-3/4 transcription factor has been demonstrated to be critical to the degree of differentiation of a cell. Only an optimal level of Oct-3/4 expression will maintain ES cells in their undifferentiated state (Niwa et al., 2000). Oct-3/4 has a high binding affinity for another transcription factor, Sox-2 (sex-determining region Y box 2). Oct-3/4 and Sox-2 bind heterodimerically to each other, and also stimulate transcription in a feedback loop type manner by binding to their opposing number's encoding gene (Ambrosetti et al., 2000, Catena et al., 2004, Chew et al., 2005) (Okumura-Nakanishi et al., 2005) (Tomioka et al., 2002). It is possible that Sox-2 acts more to monitor and modulate levels of Oct-3/4, as opposed to the direct activation of transcription factors

themselves. This theory is supported by Sox-2 inducible-knockdown experiments (Masui et al., 2007).

Alternative splicing of Oct-3/4 pre-mRNA yields two isoforms, the longer Oct-3/4A and the shorter Oct- 3/4B isoforms. Studies have suggested that is it the Oct-3/4A isoform, that contains an additional N-domain sequence, that is responsible for the pluripotency induction in ESCs (Lee et al., 2006).

2.1.4.2 Nanog

The *NANOG* gene and translated protein, which was named after a legendary immortal Scottish hero, has been called the master pluripotency gene (Chambers et al., 2003, Chambers et al., 2007, Hart et al., 2004). The protein itself contains a homeodomain to facilitate DNA binding. Nanog mRNA can be detected in mouse and human ESC lines, but never in their differentiated progeny (Chambers et al., 2003). It is also detected in the inner cell mass of a developing embryo (Mitsui et al., 2003). If the levels of Nanog protein are increased by genetic manipulation, the need for Oct-3/4 activity can be negated (Chambers et al., 2003). Nanog appears to head the hierarchy of transcription factors that control pluripotency: *Nanog*-deficient ESCs lose pluripotency and *nanog*-deficient ICMs failed to develop to the epiblast stage (Mitsui et al., 2003).

Interaction of the Oct-3/4:Sox-2 complex with the Nanog promoter has been demonstrated, and a study of the promoter sequences bound to by Oct-3/4, Sox-2 and Nanog demonstrated considerable overlap in target genes, although binding sites themselves differed (Loh et al., 2006). Over 350 genes were found to be co-occupied by all three transcription factors including the

Pou5f1, *Sox-2* and *Nanog* genes themselves, and also many genes found in an inactive state in ESCs. This would suggest the three factors serve to repress germ layer differentiation by muting expression of these genes (Boyer et al., 2005), and also form a positive feed-forward loop to regulate their own expression.

Nanog is also implicated in many other signalling pathways within the cell too. For example, Nanog expression can be up-regulated by Oct-3/4-bound ß-catenin (Takao et al., 2007).

2.1.5 Glycolipids and protein expression

Similarly, the panel of proteins whose expression has become inseparably linked with undifferentiated ESCs line has been devised.

Immunocytochemical data will often report expression of TRA 1-60 and TRA 1-81 (tumour recognition antigens 1-60 and 1-81) and SSEA-1, -3 and -4 (stage specific embryonic antigens 1, 3 and) to characterize an ESC line (Henderson et al., 2002, Sato et al., 2003). TRA 1-60 and TRA 1-81 refer to two separate binding sites on the same molecule. TRA 1-60 and TRA 1-81 monoclonal antibodies demonstrate high binding affinities for two epitopes on a high molecular weight, heavily glycosylated protein. The glycoprotein is localised to the cell surface by function of its transmembrane proteinaceous domain and is thought to participate in cell adhesion functions. SSEA-3 and SSEA-4 are glycolipids with a carbohydrate core which are localised to the cell surface.

2.1.6 Other aspects of hESC biology

2.1.6.1 hESCs and chromosomal instability

Using data available from whole genome sequencing, the human genome mutation rate is estimated to be ~1.1×10^{8} per site per generation (Roach et al., 2010). ESCs in culture are also subject to this mutation frequency, however due to their capacity for infinite cell division, ESCs have a greatly increased probability of acquiring a mutation during culture. In addition, there is a strong selection that is aimed at increasing the probability of self-renewal (Andrews et al., 2005). Indeed, hESC lines often show progressive 'adaptation' to culture, with the result that late-passage cells may be maintained more easily (Enver et al., 2005).

Acquisition of mutations by hESCs obviously impacts on their potential use for patient-centred therapies. Some warning signs reminiscent of neoplastic cells, such as growth factor independence, and a particular type of chromosomal staining associated with cancer cells have been reported in hESCs (Baker et al., 2007, Werbowetski-Ogilvie et al., 2009). Studies of karyotypic aberrations have shown chromosomal gains on chromosome 17 in three independent hESC lines, with occasional gain on chromosome 12 (Draper et al., 2004) In the case of trisomy in chromosome 12, morphological changes in hESC colonies are dramatic and can be easily visualized by eye (Seol et al., 2008). *NANOG* has been suggested as a candidate gene on chromosome 12p which, when overexpressed promotes the self-renewal of hESCs (Darr et al., 2006, Chambers et al., 2003). A minimal amplicon in chromosome 20q11.21, which most likely corresponds to the anti-apoptotic

gene, *BCL2L1*, has also been identified (Amps et al., 2011). The amplification of this region promotes the survival of ESCs *in vitro,* thereby providing a strong growth advantage.

Correlations between tissue culture techniques and genetic abnormalities have been suggested (Yang et al., 2008, Mitalipova et al., 2005). Therefore it is imperative that hESCs culture is optimized to sustain chromosomal stability and normal karyotype.

2.1.6.2 hESCs and epigenetics

Epigenetics refers to functionally relevant modifications to the genome that do not involve a change in the nucleotide sequence of DNA. Methylation and acetylation are perhaps the most studied epigenetic methods, but other epigenetic modifications include ATP-dependent chromatin remodelling, exchange of histone variants, ubiquitination, phosphorylation and the involvement of small RNA molecules (Gan et al., 2007). Histones can package genomic DNA in a slightly different manner, according to epigenetic modifications, and therefore affect the probability of transcription of the genes occurring.

Studies of epigenetic processes in ESCs revealed that promoter regions demarcated with a specific histone 3 methylation modification were transcriptionally active, including the promoters of the well-established hESC marker genes *Nanog, Oct-3/4* and *Sox-2* (Ng et al., 2008, Zhao et al., 2007). When two repressive trimethylation events were mapped across the entire hESC genome, genes with these modifications were often found to be key regulators of developmental function (Pan et al., 2007). Interestingly,

epigenetic modifications observed on the *Nanog, Sox-2* and *Oct-3/4* genes appeared to switch from activating to repressive events, upon hESCs differentiation. A substantial degree of epigenetic stability over long-term culture of hESCs has been suggested as the imprinted genes have been shown to retain their monoallelic expression (Rugg-Gunn et al., 2005).

The importance of culture conditions of hESCs is highlighted by the importance of low oxygen (5% pO_2, 36 mm Hg) for derivation and propagation of hESCs (Lengner et al., 2010). Functional gain of the X chromosome, resulting from loss of X-chromosome inactivation in culture-adapted ESCs with two karyotypically normal X chromosomes has been reported (Enver et al., 2005). On the contrary, physiological levels of oxygen mimic the uterine environment and prevent X chromosome inactivation (Lengner et al., 2010). The presence of two active X chromosomes aids in maintaining pluripotency and suppressing spontaneous differentiation by maintaining hESCs in a more immature state.

2.1.6.3 hESCs, telomerase and telomere length

Telomerase is an enzyme that adds specific six nucleotide DNA repeats to the 3'-end of telomere regions, situated at the end of chromosomes. In human cells, there are around two thousand of these repeats in the average telomere. During each round of DNA replication, a small piece at the end of telomere is lost, owing to the incapacity of normal DNA polymerases to copy the very ends of chromosomes (Harley et al., 1990, Blasco et al., 1997). In such a scenario, the chromosomes would become progressively shorter with each replication, and the coding information contained with the

chromosomes could be deleted. Telomerase therefore functions to prevent this happening, by lengthening telomeres and providing non-essential genetic material that can be sacrificed to the process of replication, without the loss of any genetic material (Harley et al., 1990).

Growth arrest in hESCs has been reported to occur when telomeres are at a length similar to that seen in senescent cells (Forsyth and McWhir, 2008). The starting telomere length of hESC-derived progenitors also exerts effects on the proliferative potential of progeny populations.

When hESCs were transduced with hTert (the active subunit of telomerase) immortalisation of progenitor cell populations was achieved (Forsyth and McWhir, 2008). In a study using iPSCs derived from old and young patients, the iPS colonies were found to contain cells with telomere lengths longer than that of the parental cells, indicating that reprogramming stimulates telomere elongation (Marion et al., 2009b).

2.1.7 Human ESCs and the epithelial-mesenchymal transition

Human ESCs have apical-basal, epithelial-type polarity with functional tight junctions and microvilli localized to the apical regions, and asymmetric distribution of organelles (Krtolica et al., 2007, Van Hoof et al., 2008). In addition expression of epithelial proteins primarily belonging to epithelium-related cell-cell adhesion complexes, including adherens junctions, tight junctions, desmosomes, and gap junctions, has been reported in hESCs (Huettner et al., 2006, Baharvand et al., 2006, Wong et al., 2004).

An epithelial-mesenchyme transition (EMT) is a phenomenon observed at several points during embryogenesis, when cells epithelial cells lose their polarity and become motile mesenchymal cells, in order to form additional parts of the embryo. EMT events have been documented to occur in the neural tube, primitive streak and developing heart during human embryogenesis (Ahlstrom and Erickson, 2009, Baum et al., 2008, Duband et al., 1995). In hESC colonies an early differentiation process has been identified, whereby epithelium-type cells inside or on top of colonies seem to alter their morphology to mesenchymal-type cells, and migrate to the periphery of the colonies (Ullmann et al., 2007). This process is reminiscent of EMT and expression of the EMT-associated genes *Snail* and *Slug* are both up-regulated in the periphery of colonies examined, in correlation with a switch in expression from epithelially-associated E-cadherin to mesenchymally-associated vimentin (Ullmann et al., 2007). Increased immunopositivity for brachyury through early differentiation of hESC colonies mirror those of vimentin, again in combination with elevated levels of *Snail* expression (D'Amour et al., 2005). Brachyyury is a transcription factors that defines the midline of a bilaterian organism and mesoderm formation during gastrulation (Edwards et al., 1996). These data suggest that differentiating hESC cultures include features of an EMT, similar to that observed during vertebrate gastrulation.

2.1.8 *In vitro* differentiation of hESCs: embryoid bodies formation

Embryoid bodies are three-dimensional clusters of cells, formed from embryonic stem cells cultured in suspension. These clusters which mimic many of the hallmarks of early embryonic development acquire molecular markers specific to the three embryonic germ layers (Itskovitz-Eldor et al., 2000). Following cell aggregation, a layer of primitive endoderm forms spontaneously on the exterior surface of the embryoid bodies (Maurer et al., 2008). The cells compromising this primitive endoderm exhibit an epithelial morphology, further differentiate into visceral and parietal endoderm, and deposit a basement membrane rich in laminin and type IV collagen (Li et al., 2001). As the development of embryoid bodies progresses, differentiated cell phenotypes of all three germ lineages can be seen. For example, evidence of haematopoietic differentiation of embryoid bodies is supported by the appearance of yolk sac-like blood islands and spontaneously contractile foci of cells within embryoid bodies, which indicates cardiomyogenic differentiation (Doetschman et al., 1985). Elongated cell projections resembling neurite extensions can be seen radiating out from embryoid bodies upon plating onto adherent substrates.

Therefore, embryoid body formation is commonly used to initiate spontaneous differentiation which can then be manipulated and directed towards a specific cell lineage. For example, VEGF is known to direct differentiation within embryoid bodies to mesenchymal fate which is marked by expression of the mesenchymal intermediate filament protein vimentin (Choi et al., 1998).

2.1.9 *In vivo* differentiation of hESCs: teratoma formation

Evaluation of the capability of a ESC line to differentiate into any tissue within an organism, would optimally be performed by carrying out blastocyst chimerism, and then allowing the embryo to develop through the full gestation time. Indeed, mouse ESCs are assessed in this manner (Buehr et al., 2008, Ueda et al., 2008). Due to obvious ethical reasons, this technique cannot be extrapolated to prove pluripotency of hESCs using human blastocysts.

Instead the teratoma assay can be applied. Transplanting undifferentiated hESCs into permissive sites of mice and analysing the resultant tumour, or teratoma, provides an indication of the spontaneous differentiation capabilities of an hESC line. In a teratoma assay, a true hESC line will differentiate into tissue types from each of the three extra embryonic germ layers, the ectoderm, endoderm and mesoderm. This is usually assessed by recognising cell types by morphology, in H&E stained sections of the teratomas.

The teratoma assay is compulsory for characterization of any new hESC line (Brivanlou et al., 2003). Cells are transplanted sub-cutaneously, intra-testicularly or in some cases into the liver lobes of immune-deficient mice (Thomson et al., 1998, Cooke et al., 2006, Gertow et al., 2004, Heins et al., 2004, Reubinoff et al., 2000, Stojkovic et al., 2004). These injection sites are chosen for the ease of delivery of the cells into the correct location, and for the confinement of the cells in that location once delivered. Delivery of the cells into the liver lobes has been reported to generate teratomas containing more immature cell types, in comparison to sub-cutaneous or intra-

testicular injection, and also to promote more vascularisation within the tumour (Lees et al., 2007). Three-dimensional scaffolds and other substances may be co-injected with the hESCs, to improve teratoma formation (Lees et al., 2007, Przyborski, 2005).

The teratoma assay is not only proof of *in vivo* differentiation but is also a proof that the cells are not cancerogenic. Culture-adapted transformed hESCs have been shown to form more aggressive tumours, called teratocarcinomas, the malignant counterparts of teratomas (Werbowetski-Ogilvie et al., 2009, Blum and Benvenisty, 2009).

2.1.10 Development of hESC Culture Systems

The establishment of the first hESC lines was achieved by using mouse embryonic fibroblasts (MEFs) as feeders and foetal bovine serum (FBS)-containing culture medium (Thomson et al., 1998, Reubinoff et al., 2000) and many laboratories continue to use them routinely for long-term hESC culture. Feeder cells such as MEFs have a capacity to support the growth and proliferation of hESCs while maintaining them in an undifferentiated state. The exact mechanism of how feeder cells support hESCs growth is not understood, but it is likely that feeders provide both a suitable attachment substrate through cell membrane proteins and essential soluble growth factors. MEFs are primary cells derived from day 12.5–14.5 murine foetuses, so they are used optimally between passage 3 and passage 6. To prepare feeder layer, MEFs are mitomically inactivated either by γ-irradiation or mitomycin C treatment.

Despite the effectiveness of feeder-supported culture of hESCs, the continued use of feeders and animal- derived components in hESC cultures will hinder the development of clinical applications due to: a) the presence of immunogenic material (Martin et al., 2005); b) the risk of transmitting animal virus or prion material; and c) difficulty with quality control of these undefined components. Furthermore, feeder-dependent culture of hESCs calls for manual passaging which is both labour-intensive and time-consuming. This in turn presents challenges in large-scale expansion of hESCs and limits the efficiency and reproducibility of differentiation protocols. Therefore, an important advancement in the field is the definition of culture conditions that support the proliferation and culture of hESCs without the need for feeders or animal-derived components. An optimal culture system would include an appropriately buffered medium that contains all metabolites, cytokines and growth factors required for self-renewal and survival of undifferentiated hESCs as well as a culture matrix that supports cell growth.

First optimizations in hESCs culture aimed at replacing animal feeder cells with various alternative human cells, such as HFFs (Amit et al., 2003, Hovatta et al., 2003), fetal placental fibroblasts (Genbacev et al., 2005), uterine endometrium cells (Lee et al., 2005), adult marrow stroma cells (Cheng et al., 2003), fetal/adult fallopian tube-epithelial cell origin (Richards et al., 2002, Richards et al., 2003). Feeder cells are routinely cultured in medium containing FBS which raises an issue of contaminating hESCs with animal molecules from serum. To avoid this, several groups have reported methods for culturing feeder cells with human serum (Amit et al., 2003,

Wang et al., 2005), but this approach still does not eliminate batch-to-batch variation introduced by the presence of serum. Consequently, several serum- and feeder-free culture conditions have been developed (Xu et al., 2001, Levenstein et al., 2006, Amit et al., 2004, Vallier et al., 2005, Lu et al., 2006).

One of the most successful culture conditions has been reported by Xu *et al* (Xu et al., 2001). This culture system utilized BD Matrigel™ as a culture matrix and MEF conditioned medium supplemented with bFGF. Matrigel is a complex mixture of mouse sarcoma origin that contains extracellular molecules, such as laminin, type IV collagen, heparin sulphate and proteoglycan (Kleinman et al., 1986). Under the conditions described, hESCs were maintained for over ~130 population doublings and retained all the morphological and molecular characteristics of undifferentiated hESCs, as well as normal karyotypes. The cells also retained their pluripotent capacity and consistently differentiated into many types of cells including neurons, hepatocytes, and cardiomyocytes.

2.1.11 Defined media for hESC culture

As mentioned above, hESCs were traditionally cultured in FBS (Thomson et al., 1998, Reubinoff et al., 2000), which was later replaced with human serum (Richards et al., 2002). To overcome serum-related variability, several groups have optimized serum-free hESCs culture using serum replacement (KnockOut™ Serum Replacement, Ko-SR, Invitrogen) (Amit et al., 2000, Koivisto et al., 2004, Stojkovic et al., 2004). Despite its

effectiveness Ko-SR is undefined and contains animal components. Consequently, two groups have reported the culture of hESCs in chemically defined media (Liu et al., 2006b, Vallier et al., 2005, Yao et al., 2006), although none of these methods could be considered xeno-free due to the presence of bovine serum albumin (BSA) and xeno-based matrices. Other successful feeder- and serum-free cultures have been using ECM and a combination of transforming growth factor ß (TGFß) and bFGF (Amit et al., 2004), or or high levels of bFGF alone (Levenstein et al., 2006). Successful propagation of hESCs has been achieved in xeno-free and defined culture media with fibronectin/collagen matrix (Lu et al., 2006). "TeSR" is a serum-free, xeno-free medium that was shown to support derivation and long-term feeder-independent culture of hESCs, and was developed by Ludwig and colleagues at the WiCell Research Institute (Madison, WI) (Ludwig et al., 2006b). The formulation of "TeSR" included high levels of bFGF, together with TGFß, γ-aminobutyric acid, pipecolic acid, and lithium chloride. The original publication described the use of cell support matrix composed of type IV collagen, fibronectin, laminin, and vitronectin of human origin (Ludwig et al., 2006a). Subsequently, STEMCELL Technologies has developed mTeSR™1 as standardized media for feeder-independent maintenance of hESCs in culture. This culture medium is complete, serum-free, defined formulation based on the publications by Ludwig *et al* (Ludwig et al., 2006a, Ludwig et al., 2006b). It contains a bovine albumin source that supports the long-term, feeder-independent culture of hESCs and has also been used for successful feeder-free derivation of iPSCs (Yu et al., 2007, Sun et al., 2009). The culture conditions are optimal when BD Matrigel hESC-

qualified Matrix (BD) is used as a substrate. Each batch of this matrix is pre-qualified to ensure consistency, reproducibility, and reliability in performance. Such culture system for hESCs ensures consistent conditions due to the elimination of undefined medium components and removal of the inherent variability associated with feeder cells and conditioned media. Standardization of culture methods also dramatically increases reproducibility of data.

Therefore, I opted to adapt the KCL-002 hESC line to grow on Matrigel substrate in the presence mTeSR™1 medium. To prove that the adaptation was successful it was important to show that the cells remained phenotypically homogeneous and karyotypically normal, expressed high levels of multiple antigens associated with pluripotency as well as genes responsible for their pluripotent status. It was also important to show that the cells could be differentiated *in vitro* and formed teratomas containing derivatives of endo-, meso-, and ectodermal lineages.

2.2 METHODS

2.2.1 Mouse embryonic fibroblast culture

Undifferentiated hESCs are traditionally grown on feeder layer of mouse embryonic fibroblasts (Thomson et al., 1998).

MEFs were derived from embryonic day 16 embryos derived from C57/BL6 male mice crossed with 129/SV female mice, prior to the commencement of this study.

Early passage (P1-P2) MEFs were cultured in 175cm² tissue culture flasks (Appleton Woods, Birmingham, UK) in an atmosphere of 5% CO_2 until 70% confluence was reached. Flasks were coated with filtered 0.1% (w/v) gelatin solution (Sigma, Gillingham, UK) prior to addition of the cells to facilitate cell adherence (5min at room temperature). Excess gelatin-containing solution was aspirated away.

MEF culture medium comprised High Glucose Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Paisley, UK), 10% embryonic stem cell-tested foetal calf serum (ES-FCS; Autogen Bioclear, Calne, UK), 0.1mM non-essential amino acids (NEAA; Invitrogen), 2mM Glutamax (Invitrogen) and 0.1mM 2-ß-mercaptoethanol (Sigma). All medium components were filtered through a 0.2µM filter system (Appleton Woods) to ensure sterility.

2.2.2 Preparation on MEFs feeder-layers

Passage 3 MEFs at 70% confluence were used to make 'feeder plates' for the maintenance of hESCs. All feeder plates were made using MEFs derived from the same batch to ensure consistency in the cellular environment of the plates.

MEFs in tissue culture flasks were washed with tissue-culture grade phosphate-buffered saline (PBS; Invitrogen) and incubated with the mitomycin C mitotic inactivation reagent (Sigma) at a working concentration of 5µg/ml at 37°C for 2.5 hours. Mitomycin C cross links DNA, thereby inhibiting chromosome rearrangement necessary for mitosis. Mitomycin C solution was removed by aspiration, the cell monolayer washed

with PBS and cells released from the culture vessel surface enyzymatically, by incubating with TrypLE Express (Invitrogen) at 37°C for 4min. To neutralize the enzyme activity, an equal volume of MEF medium was added to the cell solution and the cells were collected by centrifugation at 400g for 5min. Meanwhile, wells of 4-well plates (VWR, East Grinstead, UK) were gelatinised as described above, and 500µl MEF medium added per well.

Collected MEFs were counted using a haemocytometer (Hausser Scientific, Horsham, PA, USA) and 3.2x10 /cm² viable inactivated MEFs per well were added. Plates were agitated gently to disperse MEFs evenly through the well and incubated overnight to allow for efficient cell attachment. Plates were used within 3 days of this time point.

2.2.3 Buffalo rat liver cell culture

Survival and growth of human embryonic stem cells is greatly enhanced by culture in media that has been partially 'conditioned' by a previous cell population, to release chemical mediators and growth factors into the medium. Buffalo Rat Liver cells (BRL cells, gift from Prof. Paul Sharpe, King's College London) were used for this purpose.

BRL cells were cultured in 175cm² tissue culture flasks, in medium composed of High Glucose DMEM, 20% ES-FCS, 2mM Glutamax, 0.1mM NEAA and 0.1mM 2-ß-mercaptoethanol (Invitrogen) ('BRL medium'). Medium components were filter-sterilised prior to use.

'BRL conditioned medium' (BRLCM) was collected from flasks at 70% confluency and stored at -80°C until use. Storage at -80°C was necessary to

protect the biological activity of active components of the medium. BRL cells were passaged enyzymatically using TrypLE Express, and re-seeded into new tissue culture flasks at a ratio of 1:10 until 70% confluence was achieved again. Culture technique was maintained in this fashion to generate large quantities of BRLCM.

2.2.4 Maintenance of hESCs on MEF feeder layers

For optimum aseptic techniques, work with hESCs was performed wearing surgical masks and shoe covers (Kimberley Clark, West Malling, UK) in addition to lab coats and gloves.

The hESC line used in this study (KCL-002) was derived in the Stem Cell Biology Laboratory at King's College London with Guys and St Thomas' Hospital ethical approval under HFEA license R0133, and with a full informed patient consent. Colonies of KCL-002 hESCs were maintained in an undifferentiated state by a combination of culture on inactivated MEF feeder plates and exposure to the bFGF in culture medium (Pickering et al., 2005, Pickering et al., 2003). hESC medium was composed of a 1:1 ratio of BRL medium and BRLCM and 8ng/ml bFGF (Peprotech, London, UK).

The medium was replaced every other day (500µl) and supplemented on alternate days (250µl). Every 7-10 days the colonies were mechanically passaged by 'cutting' undifferentiated cell colonies into small clumps under a dissection hood, using Pasteur pipettes (Sigma) pulled to a fine point in a Bunsen flame. An average hESC colony was cut into 10-14 pieces. Colony

sections were transferred to new wells containing fresh MEF-feeder layers and bFGF-containing medium.

The colonies were maintained at the approximate density of 300-500 cells per colony to avoid premature differentiation.

2.2.5 Feeder-free maintenance of hESCs

For feeder-free culture, KCL-002 cell line was grown on BD Matrigel hESC-qualified matrix (BD, Oxford, UK) diluted 1:100 in DMEM:Ham F-12 (Invitrogen). Small (250µl) aliquots of undiluted BD Matrigel were stored at -80°C for up to 6 months and thawed slowly on ice when required. For a 6-well plate, 1ml of diluted Matrigel was used per well. Coated plates were then left at 37°C for 1 hour before aspirating and replacing with mTeSR1 medium (Stem Cell Technologies, Grenoble, France). If not used immediately, plates were sealed with Parafilm (Appleton Woods) to prevent dehydration and stored at 4°C for up to 7 days after coating.

hESCs were passaged when the colonies were large and begin to merge, and had centres that were dense and phase-bright compared to their edges. Depending on the size and density of seeded aggregates, cultures were usually passaged 5-7 days after initial seeding.

Passaging was performed enzymatically using dispase at a concentration of 1mg/ml (Invitrogen). Stock solutions of 5mg/ml were prepared with PBS and stored at -20°C. Stock was further diluted with DMEM:Ham F-12 to working concentration and filter-sterilized prior use through a 0.22µm filter membrane.

If differentiated regions were observed, they were removed by scraping with a pipette tip. The culture medium was then aspirated and the plates rinsed with DMEM:Ham F-12. Dispase (1ml per well in a 6-well plate) was added and incubated at 37°C for up to 5min. Enzymatic activity was confirmed under phase-contrast microscope: the colony edges appeared slightly folded but most of the colony remained attached to the plate. Dispase was then removed and the plates gently rinsed twice with DMEM: Ham F-12 to dilute away any remaining dispase. DMEM:Ham F-12 (2ml per well) was then added to the plate and colonies scraped off using a cell scraper (Appleton woods). The detached cell aggregates were transferred into a 15ml conical tube and the wells rinsed with additional 2ml of DMEM:Ham F-12 to collect any remaining aggregates. The cell aggregates were centrifuged at 300g for 5min, the supernatant aspirated and pellet resuspended in mTeSR1 by gently pipetting. The split ratio of the initial cultures was 1:4. As the cells became more adapted to the new culture conditions, the split ratio was increased to 1:10. The aggregates were seeded onto a new plate coated with Matrigel.

2.2.6 Cryopreservation and thawing of cryopreserved hESCs cultured in feeder-free conditions

Only high quality hESC colonies (less than 15% of the cells being differentiated) were cryopreserved. The colonies were passaged as described above and following centrifugation gently re-suspended in mTeSR1 containing 10% ES-FCS and 10% Dimethyl sulphoxide (DMSO; Sigma),

taking care to leave the clumps larger than would normally be done for passaging. Larger clump size would ensure better survival of hESCs during cryopreservation and recovery. The suspenson was aliquoted into labeled cryovials and stored at -80°C overnight before being transferred into a liquid nitrogen vapor tank. To recover hESCs from liquid nitrogen, cryovials were quickly thawed in a 37°C waterbath until only a small frozen pellet remains. The cryovial was wiped with 70% ethanol to sterilize prior to opening. The contents were transferred into a 15ml conical tube using a 2ml pipette to minimize breakage of cell clumps. Pre-warmed mTeSR™1 was added dropwise to the tube, gently mixing as the medium is added. The cells were centrifuged at 300g for 5min. The medium was aspirated and the pellet resuspended in 5ml of mTeSR1, taking care to maintain the cells as aggregates. The colonies were then seeded onto Matrigel-coated 6-well plates. The cells were grown at 37°C, with 5% CO_2 and 95% humidity.

2.2.7 Immunofluorescent cytochemistry

To investigate the presence of molecules of interest immunocytochemical analysis was performed. The principle of immunostaining is reliant on a specific interaction of primary antibody with the target antigen and of the secondary antibody with the bound primary antibody. Immunostaining assumes both of these reactions occur with high affinity and allows the examination of cell preparations cultured *in vitro* for the expression of specific markers. For immunocytochemistry, KCL-002 hESC colonies were passaged onto 4 well plates. When hESC colonies had become established,

and before spontaneous differentiation began, cultures were washed once in PBS and incubated in 500µl of 4% paraformaldehyde (PFA; Sigma) fixative for 30min at 4°C. The wells were then washed with PBS for 5min at room temperature. For intracellular or nuclear proteins, cell membranes were permeabilised to allow access of the antibody to the relevant antigen. Where target proteins were expected to be expressed on the cell surface, permeabilsation of the cell membrane was not employed. Table 3 describes the details of antibodies used. Permeabilisation was achieved by adding 0.1% (v/v) Triton™ X-100 (Sigma) to solutions used in all steps. The nature of immunostaining relies on a specific reaction between an antibody and its cognate antigen epitope. To prevent both primary and secondary antibodies forming interactions with proteins in a non-specific manner, samples were incubated with a blocking solution for 1 hour at room temperature. The blocking solution consisted of PBS, 10% normal goat serum (NGS; Invitrogen), 1% BSA (Sigma) for all antibodies apart from anti-Sox2 antibody. Anti-Sox2 antibody was raised in goat therefore, 5% (w/v) milk powder (Marvel, St. Albans, UK) in PBS was used to block unspecific binding for this antibody. The cells were incubated with primary antibodies diluted to a working concentration (outlined in Table 3) for 1 hour, washed twice in PBS, and incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen) at 1:500 dilution for 30min. Both primary and secondary antibodies were diluted in 1% BSA in PBS. Alexa Fluor-conjugated secondary antibodies used throughout the course of this study are detailed in Table 4. Electrons in fluorophores such as the Alexa Fluor dyes absorb energy in the form of light, at a particular wavelength (the

excitation wavelength). The gained energy is released in the form of light of another wavelength (the emission wavelength), when the electrons return to the lowest possible energy level. Light emitted after excitation can be exploited when such dyes are conjugated to antibodies, to visualise immunopositivity for an antigen in cells and processed cellular samples. One drop of ProLong® Gold antifade reagent (Invitrogen) containing 4',6-diamidino-2-phenylindole (DAPI) was added to simultaneously mount and counterstain the nuclei. ProLong Gold preserves the fluorescent signal and DAPI binds to regions of double-stranded DNA rich in A and T bases and is, therefore, used to visualize the nuclei. DAPI nuclear staining elucidates a blue colour at a 350:460nm excitation: emission spectra. To allow for visualization of immunofluorescent staining glass coverslips (13 mm diameter; Scientific Laboratory Services, Wilford, UK) were put on top of the cells in the stained wells. The samples were visualised with fluorescence microscope (Nikon, London, UK). Images are taken with CCD camera, and processed with Photoshop CS8 (Adobe).

Control procedures with the omission of either the primary or secondary antibody were performed to check the specificity of the antigen: antibody interactions.

Antibody	Supplier	Host	Species reactivity	Isotype	Concentration	Antigen Localization
Anti-Oct3/4 (C-10)	Santa Cruz, Heidelberg, Germany	Mouse	Human, Mouse	IgG2b	2µg/ml	Intranuclear
Anti-Tra 1-60	Millipore, Watford, UK	Mouse	Human	IgM	8µg/ml	Extracellular
Anti-SSEA-4	DSHB, Iowa City, USA	Mouse	Human	IgG3	1:50 dilution	Extracellular
Anti-Sox2 (Y-17)	Santa Cruz	Goat	Human, Mouse, Rat	IgG	1µg/ml	Intranuclear
Anti-Vimentin (V9)	Abcam Cambridge, UK	Mouse	Human, Rat	IgG1	5µg/ml	Intracellular

Table 3 Primary antibodies used in Chapter 2.

Secondary antibody	Conjugation	Excitation wavelength	Emission wavelength	Light colour produced
Goat anti-Mouse IgG	Alexa Fluor 488	488nm	520nm	Green
Goat anti-rabbit IgG	Alexa Fluor 488	488nm	520nm	Green
Goat anti-mouse IgG	Alexa Fluor 568	575nm	600nm	Orange-red
Goat anti-rabbit IgG	Alexa Fluor 568	575nm	600nm	Orange-red
Donkey anti-goat IgG	Alexa Fluor 594	594nm	660nm	Red
Goat anti-mouse IgM	Alexa Fluor 488	488nm	520nm	Green

Table 4 Secondary antibodies used throughout this study.

2.2.8 Quantification of immunofluorescence data

Quantification of immunofluorescence data was carried out by counting cells showing a positive fluorescence signal for Nanog and Oct3/4 in 3 independent fields containing 100 cells. Data are presented as percentage of cell numbers in relation to the total number of DAPI-positive cells. Quantification was carried out in 3 independent experiments. Values are presented as mean ± Standard Error of the Mean (SEM). Inferential error bar was used in this analysis in order to more accurately interpret the data. SEM= SD/√n and represents a measure of how variable the mean will be, if the study is repeated many times.

2.2.9 Quantitative polymerase chain reaction

To study the expression levels of gene of interest quantitative real-time polymerase chain reaction (quantitative PCR) technique was used. When a gene is transcribed from genomic DNA sequences, a chain of nucleotides encoding a specific amino acid sequence is generated, termed messenger ribonucleic acid (mRNA). Presence of mRNAs corresponding to a particular gene in a sample is used to infer the transcriptionally active status of that gene. mRNA molecules are extremely unstable and particularly vulnerable to degradation by RNase enzymes, due to the open structure of the hydroxyl bonds in the ribose sugars. mRNA molecules are only ever intended to be transient information-relaying molecules. Continuous presence of mRNAs and resulting continuous translation into protein of the genetic code it holds would drain the resources of cells. DNA, in contrast, is much more stable

owing to the presence of stronger hydrocarbon bonds in the deoxyribose sugar. Therefore, a conversion of isolated RNAs into DNA-like molecules termed complementary DNAs (cDNAs) provides a more stable and malleable platform from which to study the expression levels of genes of interest.

2.2.9.1 Total RNA preparation

KCL-002 hESC colonies were harvested with dispase and centrifuged in 1.5ml microfuges at 8,000rpm for 30min. Total RNA extraction was then performed using the RNeasy kit (Qiagen, Crawley, UK). Cell membranes were lysed by mixing samples with 350µL activated Buffer RLT and homogenized by vortexing. Activation of buffer RLT involves addition of the reducing agent ß-mercaptoethanol, which irreversibly denatures RNases released by the cell during lysis, to protect the quality of purified RNA. Equal volumes of 70% ethanol were added and samples were applied to an RNeasy spin column set in a collection tube and flow-through discarded after centrifugation at 10,000rpm for 15s. Use of ethanol separates the sample into aqueous and organic phases. RNA will remain in the aqueous phase, and then become attached to the membrane of the columns. Digestion and removal of any contaminating genomic DNA was performed by a 350µl wash of buffer RW1, incubation with DNase I for 15min at room temperature and washing with RW1. Two wash steps were then performed with 500µl of RPE buffer for 15s and 2min both at 10,000rpm, and a penultimate spin in a new collection tube with no added buffer on the column at 13,000rpm for 2min. Metabolites, macromolecular components and further salts are removed in this wash step, in addition to drying the

membrane out. Addition of 20µl RNase-free water and a final centrifugation step for 1min at 10,000rpm eluted the purified RNA. Resultant total RNA was either used immediately for cDNA preparation or stored at -80°C. Purity of RNA was assessed spectrophometrically by examining the ratio of absorbance of light by sample at 260nm and 280nm. For this purpose a Nanodrop ND1000 UV-Vis Spectrophotometer (Labtech Int Ltd, Ringmer, UK) was used. Nucleotide absorbance is maximal at 260nm, whereas proteins will absorb light maximally at 280nm. Total RNA samples derived had an RNA purity ratio of between 1.8-2.0, which is widely considered good quality.

2.2.9.2 Complementary DNA preparation

Delicate RNA molecules can be converted into their more durable counterparts, DNA molecules, by the process of reverse transcription. DNA molecules generated are termed cDNAs. Levels of cDNA expression in samples were used to infer gene expression. Specialised primers directed against the poly(A) tail found at the end of mRNA molecules can be used as a selection tool to ensure only mRNA molecules are reverse transcribed: 4µg Oligo d(T)18 (Molecular Biology Unit, King's College London, UK), 0.5mM PCR nucleotides (Promega, Southampton, UK) and between 2-6µl total RNA were combined in sterile PCR tubes. Volume was brought to 14µl with analytical grade water and the sample was incubated at 70°C for 3min to denature RNA secondary structures and then quickly placed on ice to allow annealing of primers to RNA. After that 4µl of 5X AMV buffer, and 40 units of RNase inhibitor (both Promega) were added to all samples. 10 units of

AMV reverse transcriptase (Promega) or 1µl dH2O were added to experimental or control samples respectively to make the final 20µl volume. Addition of distilled H_2O in the place of reverse transcriptase served as a control for the presence of contaminating genomic DNA within the samples. Using a Dyad Peltier Thermal Cycler machine (Bio-Rad, Hemel Hempstead, UK), the samples were then taken through the following series of incubations: 37°C for 10min, 42°C for 30min, 52°C for 20min and finally 80°C for 10min. For quantitative PCR analysis, 20ng of cDNA samples were used.

2.2.9.3 Primer design

Oligonucleotides may be designed to anneal preferentially to sequence within genes or exons of interest. Primer pairs used in this chapter are detailed in Table 5. The Primer3 website (http://frodo.wi.mit.edu/) was used to facilitate this process. The optimal annealing and extension temperature of the DNA Polymerase contained in the SYBR® Green PCR Master Mix (Roche, Burgess Hill, UK) is 60°C, hence primers had to be chosen that were compatible with reagents to be used. Primers were designed with a maximum product size of 250 base pairs (bp). Specificity of the primer pairs for relevant genes was verified by performing nucleotide alignment searches using the Basic Local Alignment Search Tool (BLAST). All primers pairs were obtained from Sigma Genosys.

2.2.9.4 Choice of internal control

Primers were also designed against internal control gene (or 'housekeeping gene'). Levels of expression of these genes are assumed to remain constant, in situations where genes of interest vary in their levels of expression, for example following differentiation of pluripotent cells. The number of copies of housekeeping genes present in a sample after amplification is therefore used to normalize gene expression levels for sample size. For this purpose GAPDH gene was chosen.

2.2.9.5 Standard curve derivation

For each gene to be assayed, positive control cDNA samples at concentrations of 25ng/µL were used to generate standard curves. Seven serial three-fold dilutions were made from the positive control cDNA samples, and labelled dilutions A to H. Template dilutions were combined in 0.1mL tubes with quantitative PCR reagents as follows:

11.5µl Positive control template dilution

4µl 2X SYBR® Green PCR Master Mix

1.0µl relevant primers at 25ng/µl of both forward and reverse primers

Distilled H2O was added in place of template in additional tubes as a sentinel for possible contamination of reagents ('no template controls'), all dilutions of template were assayed at least in duplicate.

The Rotor-Gene 3000 Quantitative multiplexing system (Qiagen) and Rotor-Gene 6 (Version 6.0) software were used to expose samples to 3-step PCR cycles as follows:

(i) Initial denaturation: 95◦C, 10min

(ii) Denature: 95◦C, 10s

(iii) Anneal: 60◦C, 15s

(iv) Extension: 72◦C, 20s

(v) Temperature ramp: 72◦C-95◦C,

↑1◦C /5s.

Incubations (ii-iv) were repeated 40 times.

When the size of an amplicon is larger, more heat energy is required to denature the hydrocarbon bonds holding the double-stranded DNA in its helix format. The temperature ramp at the end of quantitative PCR reaction represents the temperature at which most products in a reaction became denatured, and is termed the melt curve. Plots on a melt curve peaking at the same temperature are considered to be amplicons of similar size. Rotor-Gene 6 (Version 6.0) software was used to derive standard curves by assigning dilution factors to diluents A-H of the positive control cDNA samples and correlating each with the quantitative PCR cycle at which SYBR Green-mediated fluorescence reached a threshold value (CT).

Standard curves were accepted when R^2 and efficiency values were ≥0.90 and 0.6, respectively. R^2 is a measure of the degree of linearity between the concentration of positive control cDNA template and the CT value. When a standard curve is derived for a gene, the number of quantitative PCR cycles at which analysed samples produce a CT can be used to infer copy number of the gene of interest, by way of the equation for a straight line of a graph, $y = mx+b$.

2.2.9.7 Quantitative real-time PCR

11.50µl of cDNA samples were combined with 1.0µl relevant primers at 25ng/µl of both forward and reverse primers, 4µl 2X SYBR® Green PCR Master Mix and brought to a votal volume of 20µl with distilled H_20. SYBR Green within quantitative PCR mastermix binds to double stranded DNA. The DNA-SYBR Green complex absorbs light at 488nm and emits it at 522nm. As amplicons are synthesised throughout the quantitative PCR reactions, more SYBR Green becomes bound to DNA, and hence more fluorescence is observed. The threshold cycle is used to determine the copy number of the amplicon per sample. The Rotor-Gene™ 3000 Quantitative multiplexing system and accompanying software were used to run quantitative PCRs using cycle profiles under which the standard curves for each gene were obtained. Samples were run at least in duplicate (technical replicates), and all runs included 'no template controls' as described above.

Housekeeping gene primers were also used in samples where no reverse transcriptase was added during the cDNA preparation process, as a final check for contamination by genomic DNA.

The copy number of amplicons produced by each primer set in each sample was extrapolated by importing previously acquired standard curves for the relevant genes. Quantitative PCRs for housekeeping genes were performed in parallel as normalisers, and copy numbers of all genes of interest were expressed as a ratio against housekeeping gene copy numbers.

2.2.9.8 Statistical analysis

The mean value for each data set was used to describe the central location of the data. Standard deviations (SD) were used as a measure of the dispersion of data around the mean. Analysed data was assumed to be normally distributed (Gaussian distribution). Box-and-whisker (box) plots were used to identify outlying data points (>3 SD from the mean), which were eliminated from the statistical analysis. Unpaired two-tailed Student's t-test was used to analyze data for statistical significance. Graphs and analysis were generated with GraphPad Prism (Version 5) Software. Differences in the means between two data sets were deemed statistically significant if the generated *p* value was less than 0.05.

Gene	Acronym	Primer sequence	Tm (°C)	Positive control cDNA*
Nanog	*NANOG*	Forward: 5'AATACCTCAGCCTCCAGCAGATG-3' Reverse: 5'-TGCGTCACACCATTGCTATTCTTC-3'	58.0 58.0	Human embryonic stem cell line
SRY (sex determining region Y)-box 2	*Sox2*	Forward: 5'-TGGACAGTTACGCGCACAT-3' Reverse: 5'-CGAGTAGGACATGCTGTAGGT-3'	65.4 60.7	Human embryonic stem cell line
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	Forward: 5'-GAAGGTGAAGGTCGGAGTC-3' Reverse: 5'-GAAGATGGTGATGGGATTTC-3'	60.0 60.0	Human foetal brain cell

Table 5 Details of primers used in quantitative PCR reactions in Chapter 2.
Tm = melting temperature. * These cDNA samples were used to derive standard curves for the expression of the relevant gene.

2.2.10 Directed differentiation of hESCs by embryoid body formation

Embryoid bodies are three-dimensional clusters of cells, formed from embryonic stem cells. Embryoid body cultures are used to initiate and encourage differentiation of hESCs into a wide variety of somatic cell types of all three germ layers.

Undifferentiated hESC colonies were enzymatically passaged as described above, and transferred into 60mm diameter Ultra Low Adherence Petri dishes (Appleton Woods) containing 6ml of mTeSR1 medium. Embryoid bodies are sensitive to mechanical disruption and are prone to shattering during the early stages of their formation. To minimise the rate of embryoid body loss in this way, colonies were passaged into significantly bigger pieces than for propagation purposes. No media changes were performed during embryoid body differentiation.

Differentiating embryoid bodies were harvested after 7 days of suspension culture and dissociated into single-cell suspension by passing through a 23-guage needle. The resultant cell suspension was seeded into gel-coated tissue culture plates in basic early mesoderm induction medium composed of mTeSR™1 supplemented with 10ng/ml recombinant human VEGF (R&D Systems, Abingdon, UK). VEGF was reconstituted at 100µg/ml in sterile PBS containing 0.1% BSA and stored at -20°C. The medium was changed every 2 days for 1 week before the cells were fixed and analyzed by immunocytochemistry.

2.2.11 Teratoma assay

KCL-002 hESC colonies were harvested using dispase, washed in PBS and resuspended in 100µl PBS volumes. Injections were performed using 1ml syringes and attached 27 gauge needle (both Terumo). $2x10^6$ undifferentiated hESCs were injected sub-cutaneously into NOD-SCID (Non-Obese Diabetic Severe Combined Immunodeficiency) mice. A total of 2 mice received hESC transplantations.

Mice were cared for in accordance with Home Office license guidelines and palpated regularly to monitor growth of teratomas. The susceptibility of their immune systems also dictated that the mice were housed in filter cages to prevent influx of foreign antigens. Animals were sacrificed using Schedule 2 methods at a time judged to be a humane end point. This point represented a time when discomfort of the animal had not become extreme, yet significant tumour growth had been achieved. All animal maintenance and sacrificing was carried out in compliance with Home Office guidelines.

2.2.11.1 Teratoma harvesting and processing for immunohistochemistry

After 9 weeks, tumours were harvested from sacrificed mice, washed in PBS to remove debris and submerged fully in 4% PFA at 4°C for 24 hours. Tumours were then washed in PBS and submerged in decalcifying solution composed of 4% (w/v) EDTA (VWR) in PBS for 4 days at room temperature. This allows calcium deposits in regions of bone or bone-like formation in tissues to be sequestered away from samples. Following a final wash in

PBS, tissue samples were placed in plastic cassettes (Sigma) and dehydrated by immersion in 75%, 90%, 95% and finally in 100% ethanol, using an automated tissue processor. Dehydration is necessary to permit the infiltration of paraffin wax into the tissue section. Once embedded in wax, samples were hardened by cooling to room temperature. A microtome was then employed to cut sections of 6µm thickness.

Sections were then floated on the surface of water heated to 45°C to smooth out creases, before being lifted onto positively charged glass slides (VWR) and baked at 60°C to firmly attach the sections. Wax-embedded sections and paraffin blocks were stored at room temperature until further processing.

2.2.12 Immunohistochemical analysis of teratoma sections

2.2.12.1 De-waxing prior to immunohistochemical analysis

Wax contained within the sections would repel the aqueous solutions used throughout the immunohistochemistry protocol; therefore, de-waxing steps are required prior to immunostaining. To de-wax parafin-embedded sections, slides were taken through two incubations in Xylene (5min at room temperature) and then through graded ethanol immersions, beginning with 100%, followed by 90%, 70% and finally ending with 50% ethanol (3min at room temperature each). After de-waxing, the slides were kept in tap water until staining.

2.2.12.2 Antigen retrieval

Preserving tissues involves long exposures to formalin solution, to ensure thorough infiltration of the fixative solution. Although effective in preserving tissue morphology, prolonged formalin-based fixation leads to formation of methylene bridges which cross-link proteins and therefore mask the antigens present within the tissue. Antigen retrieval methods serve to break the methylene bridges and expose the antigenic sites in order to allow the primary antibodies to bind. Antigen retrieval was achieved by submerging slides in boiling Sodium Citrate Buffer solution (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0; Sigma) and microwaving at 850W. When the solution came to the boil, the power was reduced to 650W and slides were heated for a further 20min taking care that the solution did not overboil and evaporate. The slides were then cooled on the bench top for a further 20min. This allows the slides to cool enough so they may be handled, and allows the antigenic site to re-form after being exposed to high temperature. The slides were then rinsed in gently running tap water and incubated in water for 10min. All sections were demarcated using a wax pen (VWR). Introducing wax boundaries around each section serves as a barrier to the spread of liquids within the wax circle.

2.2.12.3 Permeabilization

All incubations were carried out in a humidified chamber to avoid drying of the tissue. Drying at any stage will lead to non-specific binding and ultimately high background staining. A shallow, plastic box with a sealed lid

and wet tissue paper in the bottom was used for this purpose. Permeabilisation to allow access of the antibody to the relevant intracellular antigen was achieved with addition of 0.1% (v/v) Triton X-100 (Sigma) to Tris-buffered saline (TBS). TBS working solution (1X TBS) was made by diluting 10x TBS stock solution (200mM Tris base (VWR), 1.5M sodium chloride (Sigma), deionized water; pH 7.4) with deionized water.

2.2.12.4 Horse-radish peroxidase-mediated immunostaining

For detection of antigens of interest, horse-radish peroxidase (HRP)-mediated immunostaining was used. Firstly, endogenous peroxidase block (3% (v/v) hydrogen peroxidase (Sigma) in deionised water) was applied for 30min at room temperature to quench endogenous peroxidase activity, which may otherwise result in high levels of non-specific background staining. Hydrogen peroxide acts as a substrate for any endogenous peroxidase present, leading to its irreversible inactivation. The slides were then washed in 0.1% (v/v) Triton X-100 in TBS for 5min at room temperature. To prevent both primary and secondary antibodies forming interactions with proteins in a non-specific manner, samples were incubated with a blocking solution (10% NGS, 1% BSA in TBS) for 30min at room temperature. The slides were then incubated with pre-diluted primary antibodies for 90min at room temperature. Primary antibodies used to test for the presence of three germ layers are shown in Table 6. The slides were then washed twice in 1x TBS. In order to amplify immuno-expression signals, avidin-biotin enhancement of immunostaining was carried out. Avidin is a large glycoprotein that can be labelled with HRP. The extremely

high binding affinity of avidin for biotin was exploited in this technique. Therefore, secondary antibody incubation was carried out with an appropriately targeted biotinylated antibody for 30min at room temperature (1:250; Dako, Ely, UK). After two washes in 1x TBS, slides were incubated with Streptavidin / Biotinylated HRP complex (Vector Labs, Peterborough, UK) for 30min at room temperature and washed in 1x TBS for 5min. By additionally labelling the HRP conjugated to avidin with biotin, the formation of large complexes of HRP-avidin is encouraged, as a function of the tetravalent nature of avidin. Samples were then exposed to a solution containing diaminobenzidine (DAB), composed according to manufacturer's instructions (Vector Labs kit). DAB is a substrate for HRP, and reaction of DAB with HRP associated with the avidin/biotin complex on immobilized secondary antibodies generated an insoluble brown coloured precipitate, or black precipitate if nickel chloride was added to the DAB solution. After 10min incubation with DAB solution, the slides were washed under running tap water for 5min. Control procedures with the omission of either the primary or secondary antibody were performed to check the specificity of the antigen: antibody interactions.

Antibody	Supplier	Dilution	Localisation of target proteins
GFAP	Abcam	1:500	Ectoderm germ layer
Desmin	Sigma	1:1500	Mesoderm germ layer
Alpha-foetoprotein	Sigma	1:400	Endoderm germ layer
Keratin 14	Covance	1:1000	Basal epidermal layer
Keratin 5	Covance	1:1000	Basal epidermal layer
Keratin 18	Chemicon	1:50	Single-layer embryonic epidermis
Collagen type II	Sigma	1:80	Cartilage
Involucrin (SY5)	Sigma	1:100	Terminally differentiated epidermal cells

Table 6 Primary antibodies used to evaluate the structures observed in teratoma sections.

2.2.12.5 Counterstaing

To visualise cell nuclei, counterstaining was performed using haematoxylin (Mayer's haemalum; Sigma) for approx 90s, after which the slides were washed again under a running tap for 5min. To ensure the appropriate level of nuclear staining the slides were dipped for 5s in 0.5% HCL in 70% methylated spirit and quickly returned to water, washed for 5min and then examined microscopically. If nuclear staining was too weak, the slides were returned to Mayer's haemalum solution for a further 3min. If nuclear staining was too strong, the slides were dipped again in acid solution. Tissue sections were mounted by covering with 22x50mm coverslips (VWR), using Di-N-Butyle Phthalate in Xylene mountant (DPX; VWR). DPX is a viscous anhydrous solution containing Xylene, which protects stained samples. However, because this solution is not compatible with aqueous solutions, samples were first dehydrated by immersions in 100% ethanol followed by Xylene. The slides were allowed to dry for 30min and the sections were visualized with Zeiss Axiophot fluorescence microscope (Zeiss). Images were taken with a CCD camera (DS-U2, Nikon), and processed with Photoshop CS5 (Adobe).

2.2.12.6 Haematoxylin & Eosin Y staining of teratoma sections

The haematoxylin component stains basophilic structures, such as those with high nucleic acid content, with a blue-purple colour by binding to lysine residues. Eosin Y conversely stains eosinophilic structures pink, such as the

cytoplasm of a cell, in an electrostatic nature. After dewaxing (section 2.2.11.1), slides were immersed in a solution of Mayer's haemalum (Sigma) for 3min at room temperature and washed with deionised water. To ensure the appropriate level of nuclear staining the slides were dipped for 5s in 0.5% HCL in 70% methylated spirit and quickly returned to deionised water, washed for 5min, and then examined microscopically. If nuclear staining was too weak, the slides were returned to Mayer's haemalum solution for a further 3min. If nuclear staining was too strong, the slides were dipped again in acid solution. Eosin staining was subsequently performed by immersion in 0.5% eosin Y (VWR) staining solution for 3min at room temperature, sections washed in deionised water. The sections were mounted by covering with 22x50mm coverslips (VWR), using DPX in Xylene mountant as described in section 2.2.11.4.

2.2.12. 7 Periodic acid-Schiff (PAS) and Alcian Blue Histological stains

PAS histological stain can be used to demonstrate the presence of carbohydrates in tissue sections. The periodic acid oxidises the tissue carbohydrates containing 1-2 glycols, such as polysaccharides, mucopolysaccharides, glycoproteins and glycolipids. The oxidising reaction produces aldehyde groups, which can then condense with Schiff's reagent forming a bright red colouration and demonstrating the tissue component to which the carbohydrate is attached.

Alcian Blue, when used at pH 0.2, can be used to detect the presence of highly sulphated proteoglycans, such as those found in the extra cellular matrix of cartilage, as well as some types of mucopolysaccharides and sialylated glycocalyx of cells. The tissue parts that specifically stain by this dye become blue to bluish-green after staining.

A combined method utilising the properties of both the PAS and alcian blue methods was used to demonstrate the full complement of tissue proteoglycans in teratoma sections. The rationale of the technique is that by first staining all the acidic mucins with Alcian blue, those remaining acidic mucins which are also PAS positive will be chemically blocked and will not react further during the technique. Those neutral mucins which are solely PAS positive will subsequently be demonstrated in a contrasting manner. Where mixtures occur, the resultant colour will depend upon the dominant moiety.

Teratoma sections were deparaffinized as described in section 2.12.1. The slides were then rinsed with distilled water and immersed in alcian blue solution (pH 0.2; 1.0% (w/v) in 0.1N hydrochloric acid (VWR)) for 15min. The slides were then washed in running tap water 2min and treated with 1% aqueous solution of periodic acid (PAS kit; Sigma) for 5min at room temperature. The sections were then washed in distilled water to remove any traces of the periodic acid, and the Schiff's reagent (PAS kit; Sigma) was applied for 15min at room temperature. The slides were then washed thoroughly under running tap water, and the nuclei were counterstained, dehydrated and mounted in xylene as described in section 2.2.12.5.

2.3 RESULTS

2.3.1 Morphology of the KCL-002 hESC line cultured on inactivated MEF feeders

Undifferentiated hESCs have a very characteristic morphology that can be easily evaluated under phase-contrast microscope: colonies appear to be tightly packed with a defined border; individual cells have phase bright edges, a high nuclear/cytoplasmic ratio, and prominent nucleoli. On MEF feeders the KCL-002 hESC line has a typical morphology of undifferentiated hESCs and grows as uniform domed colonies (Figure 8). The cells are tightly packed within the colony, and maintain a defined border at the periphery of the colony. Colonies can grow on top or among the feeders, pushing away then outward as they proliferate. Differentiation is often observed as visible structure or organization, or it exhibits a darker shade, within an hESC colony. Differentiation often begins in the central part of an hESC colony. The morphology described should be preserved following adaptation to feeder-free culture.

2.3.2 Morphology of the KCL-002 hESC line adapted to growth in feeder-free conditions

During the adaptation period morphological characteristcs of KCL-002 hESC colonies were closely monitored. In the first 3 days after seeding undifferentiated KCL-002 hESCs grown on Matrigel in the presence of mTeSR1 appeared transparent and not very densely packed with cells (Figure 9). However, the density and robustness of the colonies increased

rapidly after this time point and typical hESC colony morphology was observed. The colonies continue to grow as compact, multicellular colonies, as shown in Figure 9.

The borders of colonies grown under feeder-free conditions were distinct and more pronounced compared to feeder-dependent cultures. The loss of border integrity indicated a differentiation process. Therefore, the KCL-002 hESC line has maintained the typical morphology expected of pluripotent stem cells. The next step was to confirm the expression of marker proteins.

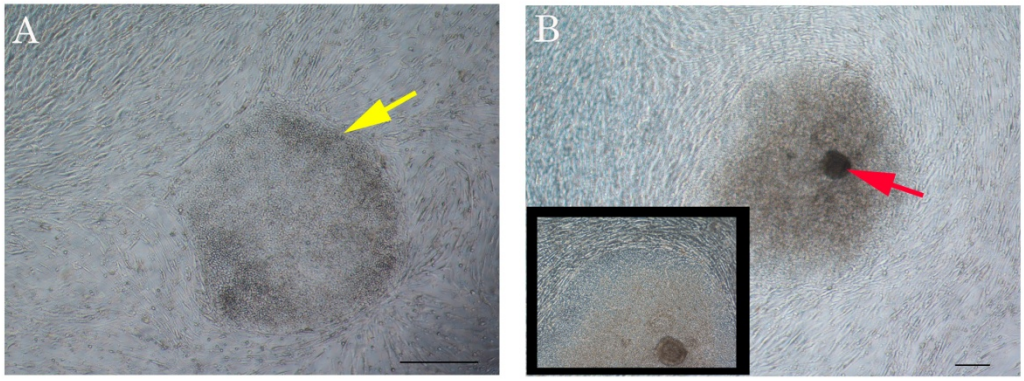

Figure 8 Morphological appearance of KCL-002 hESC colonies grown on inactivated MEF-feeder layer.
Uniform colonies growing on top (A) and amongst (B) MEF feeders. **A:** The colonies are separated from feeders by a defined border (yellow arrow); **B:** Visible differentiation can be seen in the central part of a colony (red arrow). The undifferentiated cells are tightly packed with a high nuclear/cytoplasm ratio (insert). Scale bar=100µm.

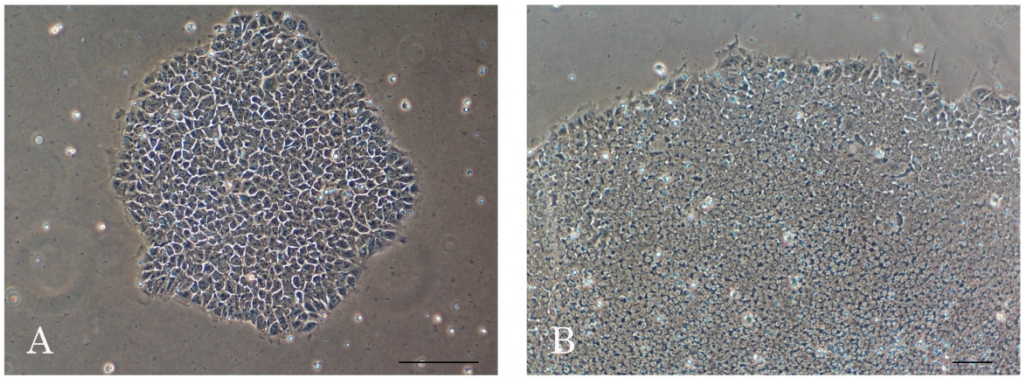

Figure 9 Morphology of KCL-002 hESC colonies under feeder-free conditions grown on Matrigel with mTeSR™1.
A: hESC colony 2 days after passaging; **B:** Large hESC colony ready to be passaged. Colonies are tightly packed with cells and have distinct borders. Scale bar=50µm.

2.3.3 Expression of pluripotency-associated markers

In order to confirm that the KCl-002 hESC line still maintained pluripotency, immunocytochemistry was performed on undifferentiated KCL-002 hESCs for 2 nuclear transcription factors Sox2 and Oct3/4 and 2 extracellular molecules Tra 1-60 and SSEA-4. Bright immunofluorescent signal and correct localization of investigated antigens suggested that KCL-002 hESCs were strongly positive for all 4 markers (Figure 10). When quantification of immunofluorescency data was carried out in three independent fields (n=3) selected at random, 92.667±2.728 and 91.000±2.646 of hESCs were found to be positive for Oct3/4 and Sox2, respectively (Figure 11). This suggests that pluripotency-associated markers are highly expressed in the KCL-002 adapted to feeder-free conditions.

The immunofluorescent analysis was consequently repeated every 10 passages for quality control, which confirmed that the KCL-002 hESC line remained highly positive for the expression of marker molecules throughout the course of this study. In order to further confirm its pluripotent status, quantitative PCR for two transcription factors was carried out.

2.3.4 Pluripotency gene expression of the KCL-002 hESCs in feeder-free conditions

Following the adaptation to feeder-free culture, the expression of two pluripotency genes, Nanog and Sox-2, was examined in the KCL-002 hESC line using quantitative PCR, and the GAPDH normalising internal control

gene (Figure 12). The analysis was carried out after 5 passages under feeder-free conditions. Data is presented as ratio of the copy number of the relevant gene of interest to the copy numbers of the normalising gene ('Gene expression ratio'). Human dermal fibroblasts (HDFs) served as a negative control. Statistical analysis revealed there was a significant difference in the expression of Nanog and Sox-2 in KCL-002 hESCs (KCL-002 Nanog 8.008 ± 3.528, Sox-2 2.350 ± 0.794) as compared to HDF controls (Nanog not detectable, Sox2 0.001 ± 0.001); *p* values were 0.0075 for Nanog and 0.0097 for Sox-2, (Mann-Whitney U test). n=5 for each cell type and gene expression.

Having confirmed the pluripotency status at mRNA level, it was important that the KCL-002 hESC line grown under feeder free conditions was indeed able to undergo spontaneous multilineage differentiation.

2.3.5 Directed differentiation of KCL-002 hESCs through embryoid body formation assays

Directed differentiation capacity of KCL-002 hESCs under feeder-free conditions was confirmed through embryoid bodies formation assay. When cultured in suspension, KCL-002 hESCs readily formed embryoid bodies which continued to grow within the time course of the experiment. Phase contrast photomicrographs of representative embryoid bodies are shown (Figure 13). When the embryoid bodies were dissociated into single-cell suspension in cultured in medium supplemented with VEGF for 1 week, vimentin-positive cells were visualized by immunofluorescence (Figure 13B).

In addition, some cells positive for brachyury transcription factor were also detected (Figure 13C), further confirming the presence of early mesenchymal cell lineages. This indicates that the KCL-002 hESC line could be directed towards differentiation into a desired cell lineage (i.e. mesoderm) *in vitro*. Spontaneous differentiation was then assessed by teratoma formation *in vivo*.

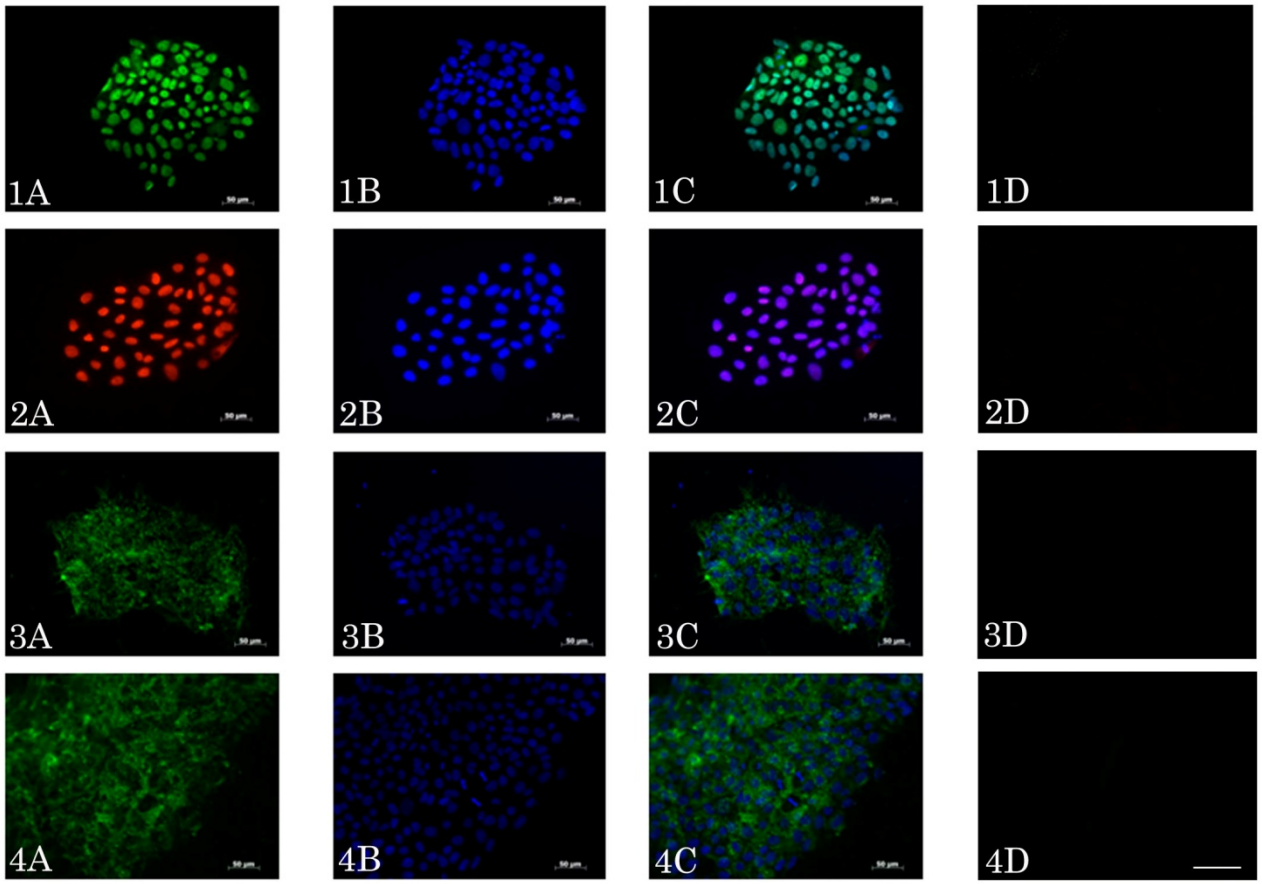

Figure 10 Expression of pluripotency-associated markers in the KCL-002 hESC line adapted to feeder-free culture.
Panel A shows the staining for the molecule of interest: four different markers were examined, from top to bottom: Oct3/4, Sox2, SSEA-4 and Tra 1-60. Panel B- DAPI nuclear staining. Panel C- merged images to show the correct localization of each molecule: nuclear localization of Oct3/4 and Sox2 transcription factors and extracellular nature of SSEA-4 and Tra 1-60 molecules. Panel D shows no primary antibody negative controls. Scale bar=50μm.

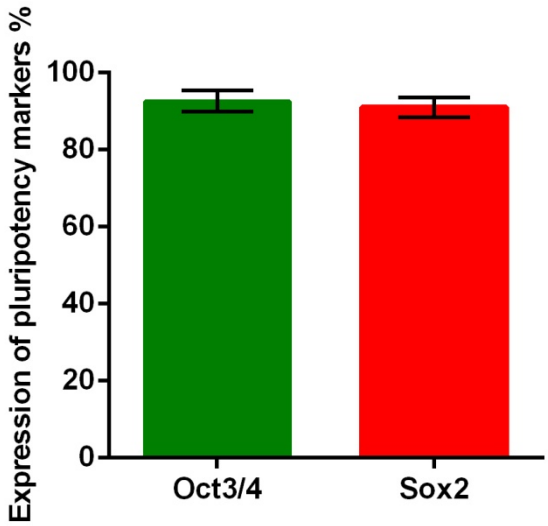

Figure 11 Quantification of pluripotency markers expression in the KCL-002 hESCs adapted to growth under feeder-free conditions.
The figure shows percentage of Oct3/4- (green) and Sox2- (red) positive cells as calculated per 3 independent fields of 100 DAPI-positive cells. n=3. Values expressed as mean ± SEM.

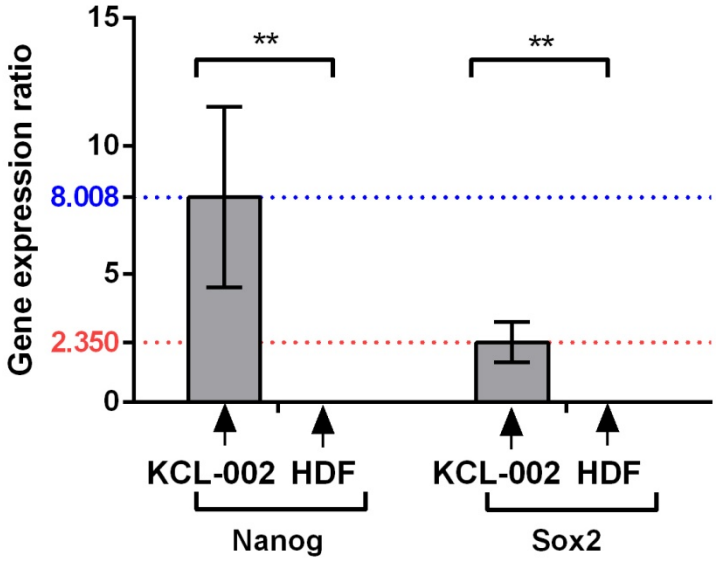

Figure 12 Expression of the pluripotency-associated genes Nanog and Sox-2 in the KCL-002 hESC line after adaptation to feeder free conditions.
Expression of the same genes was not detected in human dermal fibroblast (HDF) negative controls. Values are mean ± SD and represent the ratio of gene of interest: GAPDH transcript copy numbers. Statistical analysis demonstrated that there were significant differences in the expression of both Nanog and Sox-2 compared to HDF controls. n=5 $^{**}p < 0.01$, Mann Whitney t-test

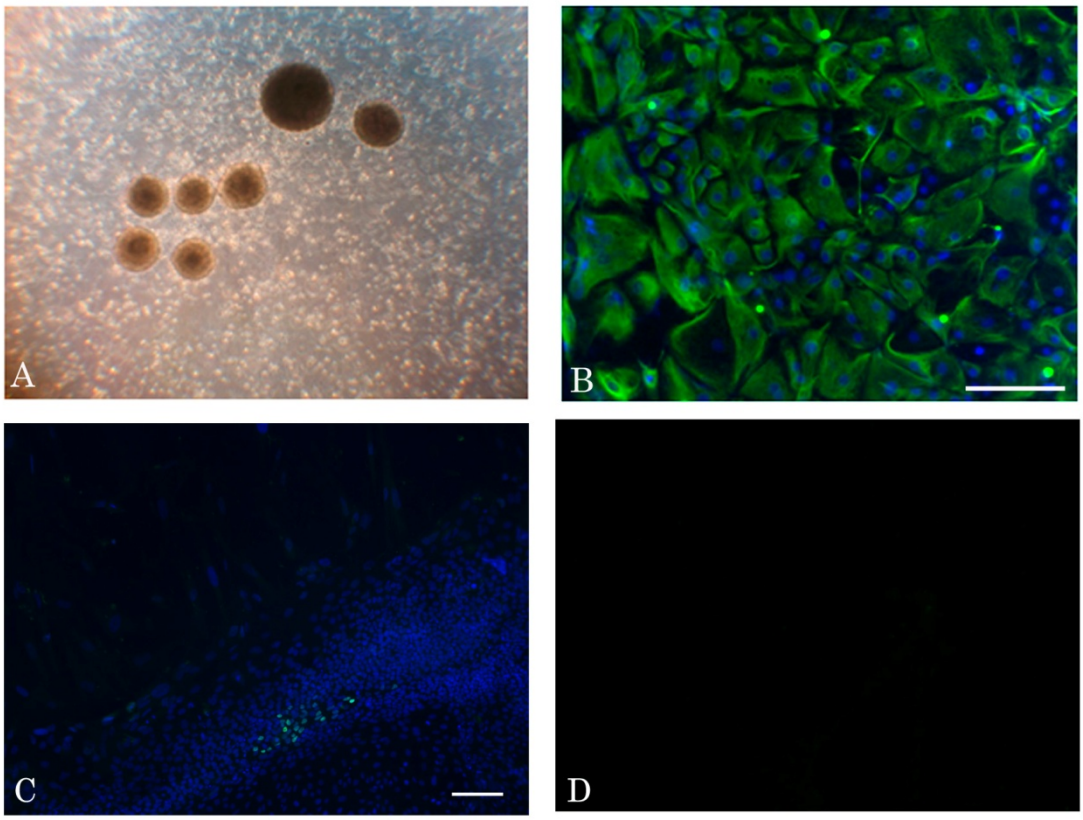

Figure 13 Evaluation of directed differentiation of the KCL-002 hESC line *in vitro* through embryoid bodies formation.
A: KCL-002 hESCs readily form embryoid bodies in suspension culture and at day 7 large aggregates are seen. B: Embryoid bodies were dissociated into single-cell suspension and cultured in basic mesenchymal induction medium for a further 7 days. The resulting cell population is positive for early mesenchymal marker vimentin (green). C: In addition, some cells were also positive for brachyury. D: No primary antibody negative control Scale bars=50μm.

2.3.6 Teratoma assay

The capacity of KCL-002 hESCs under feeder-free conditions to undergo spontaneous differentiation was confirmed through the teratoma assay. After 9 weeks, two tumours could be palpated in two NOD/SCID mice (Figure 14A). Histological examination of the tumours revealed presence of large multi-lobed cysts (Figure 14B), which had expanded from the original site of subcutaneous injection. Upon histological processing and examination of the resultant teratoma, many tissue types were immediately obvious

(Figure 15). Amongst these tissues, neuroepithelium, a derivative of the ectodermal germ layer was identified by the presence of neuroepithelial rosettes (Figure 15A). The formation of stratified epithelium was also observed (Figure 15B). Simple columnar and ciliated epithelium were also identified, both derivatives of the endodermal germ layer (Figure 15C). Smooth muscle (Figure 15D) and cartilage (Figure 16B-D) from the mesodermal germ layer were also identified.

PAS/Alcian blue staining demonstrated the full complement of tissue proteoglycans within teratoma (Figure 16). revealed the presence of "glandular epithelioid" structures containing neutral mucin (Figure 16A). These structures lie within a dense fibrous stroma that contains levels of acidic mucins that are lower than that found in the looser connective tissue surrounding the area, as indicated by a more intense blue staining. Cartilage was also identified by PAS/Alcian blue staining which showing abundance of glycogen in chondrocytes (red) and acidic mucins (blue) in the ground substance. Interestingly, a structure adjacent to cartilage highly resembled a transverse-section of a bronchus. Immunohistochemistry directed against type II collagen, the main component of cartilage, has also confirmed the presence of cartilage in the teratoma (Figure 16D). In subsequent immunohistological analysis, markers of all three germ layers were detected (Figure 17). Glial fibrillary acidic protein (GFAP) is a member of the class III intermediate filament protein family. It is heavily, and specifically, expressed in astrocytes and certain other astroglia in the central nervous system, in satellite cells in peripheral ganglia, and in non myelinating Schwann cells in peripheral nerves (Eng et al., 1971). Hence,

GFAP positivity confirms the presence of ectodermal lineages in harvested teratomas. Desmin is a type III intermediate filament found near the Z line in sarcomeres of skeletal muscle tissue, smooth muscle tissue, and cardiac muscle tissue (Capetanaki et al., 1984). Therefore, positive desmin staining indicates the presence of mesodermal tissue. Alpha-foetoprotein (α-foetoprotein) is a major plasma protein produced by the yolk sac and the liver during foetal development (Tomasi, 1977), and, therefore, serves as a reliable endodermal marker.

In order to assess the ability of the KCL-002 hESC line to undergo epidermal differentiation, a further immunohistochemical analysis was carried out against epidermal-associated proteins (Figure 18). The analysis revealed abundance of simple single-epithelium keratin 18 (Figure 18A and E), as well as basal keratins 5 and 14 (Figure 18B-D). Furthermore, minimal involucrin expression was also detected (Figure 18F), indicating terminal differentiation of hESC-derived keratinocytes in the resultant teratoma.

Collectively these results confirm that the KCL-002 hESCs line grown under feeder-free conditions is capable of differentiating into all three germ layers and producing epidermal cell lineages.

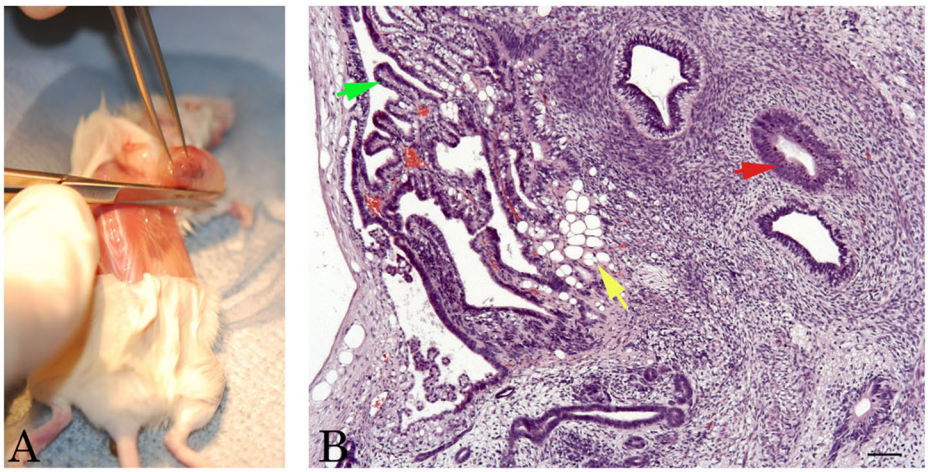

Figure 14 The appearance and histology of teratomas.
A: The appearance of subcutaneous tumour in NOD/SCID mouse; **B:** Histological analysis at low maginification reveals presence of large multi-lobed cysts. Simple columnar epithelium (green arrow), adipose tissue (yellow arrow) and presumptative neuroepithelial rosettes (red arrow) can be easily identified. Scale bar= 100μm.

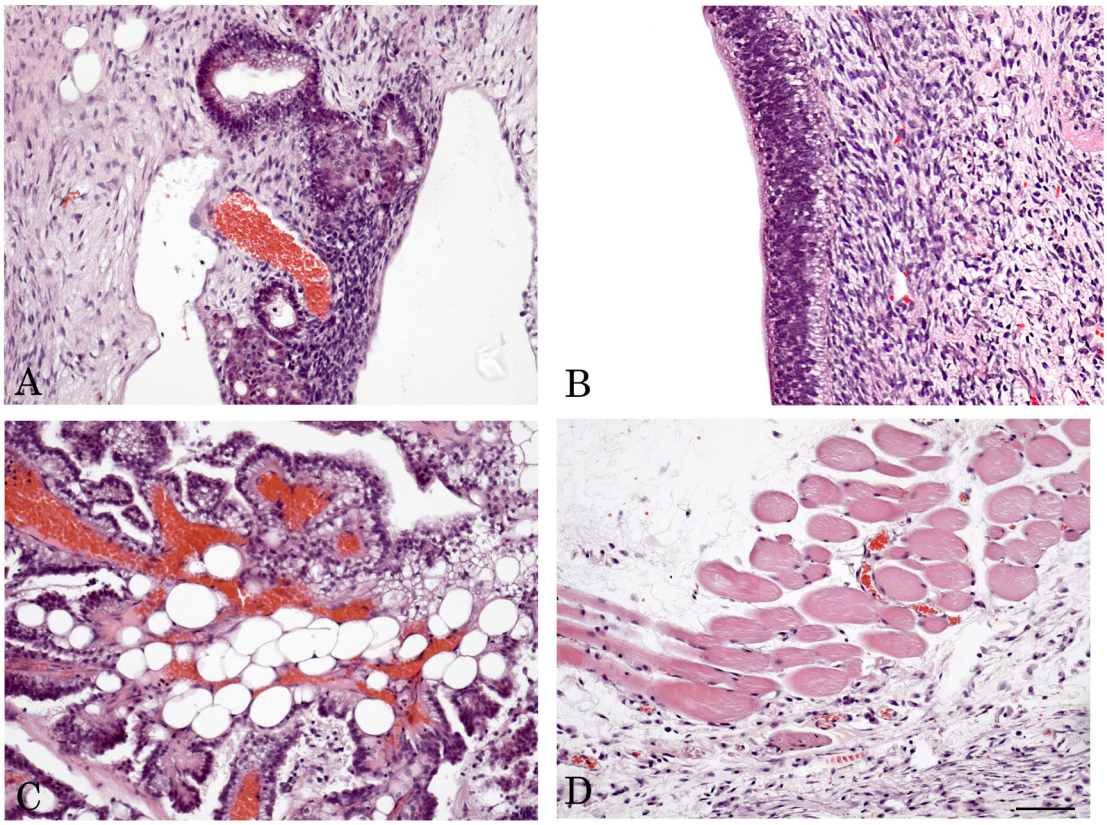

Figure 15 H&E staining of teratoma sections.
A: Presumptive neuroepithelial rosettes; **B:** Stratified epithelium; **C:** Simple ciliated and columnar epithelium; **D:** Smooth muscle. Scale bar=100μm.

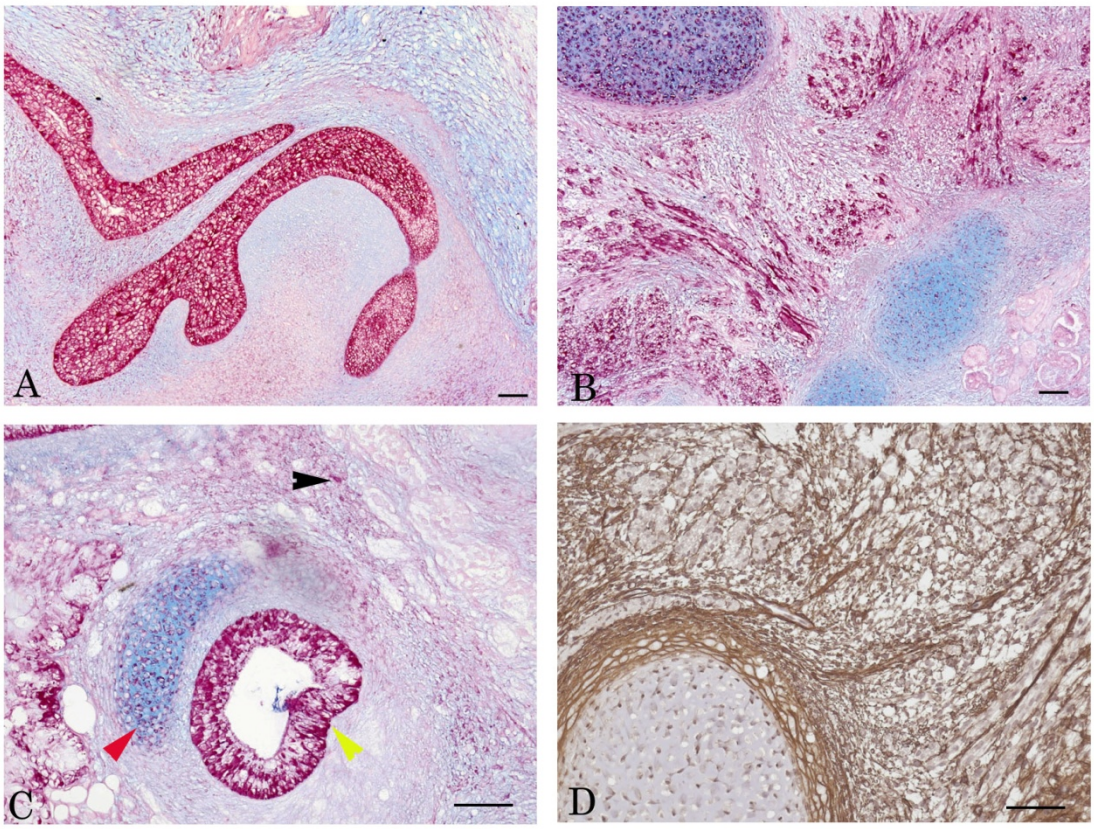

Figure 16 Histological staining of teratomas with PAS and Alcian Blue.
Acidic mucins stained blue, neutral mucins- magenta, mixtures of above- purple, nuclei- deep blue.
A: Low magnification image showing neutral mucin in glandular epitheliod structures (magenta) within dense fibrous tissue containing acidic mucins (light blue) and loose connective tissue (blue); **B:** Low magnification image showing cartilage formation within teratoma **C:** Higher magnification image showing the structure resembling a brochus. Yellow arrowhead indicates neutral mucin in epithelium, red- cartilage, black- glycogen/neutral mucins in ground substance of surrounding loose connective tissues; **D:** Collagen type II staining confirms the presence of cartilage within teratoma. Scale bars=100µm.

Figure 17 Spontaneous differentiation of the KCL-002 hESC line *in vivo* through teratoma assay.
Ectoderm formation was confirmed by Glial fibrillary acidic protein (GFAP) positivity. Mesoderm formation was validated by Desmin positivity, and α-fetoprotein confirms the presence of endodermal lineages.

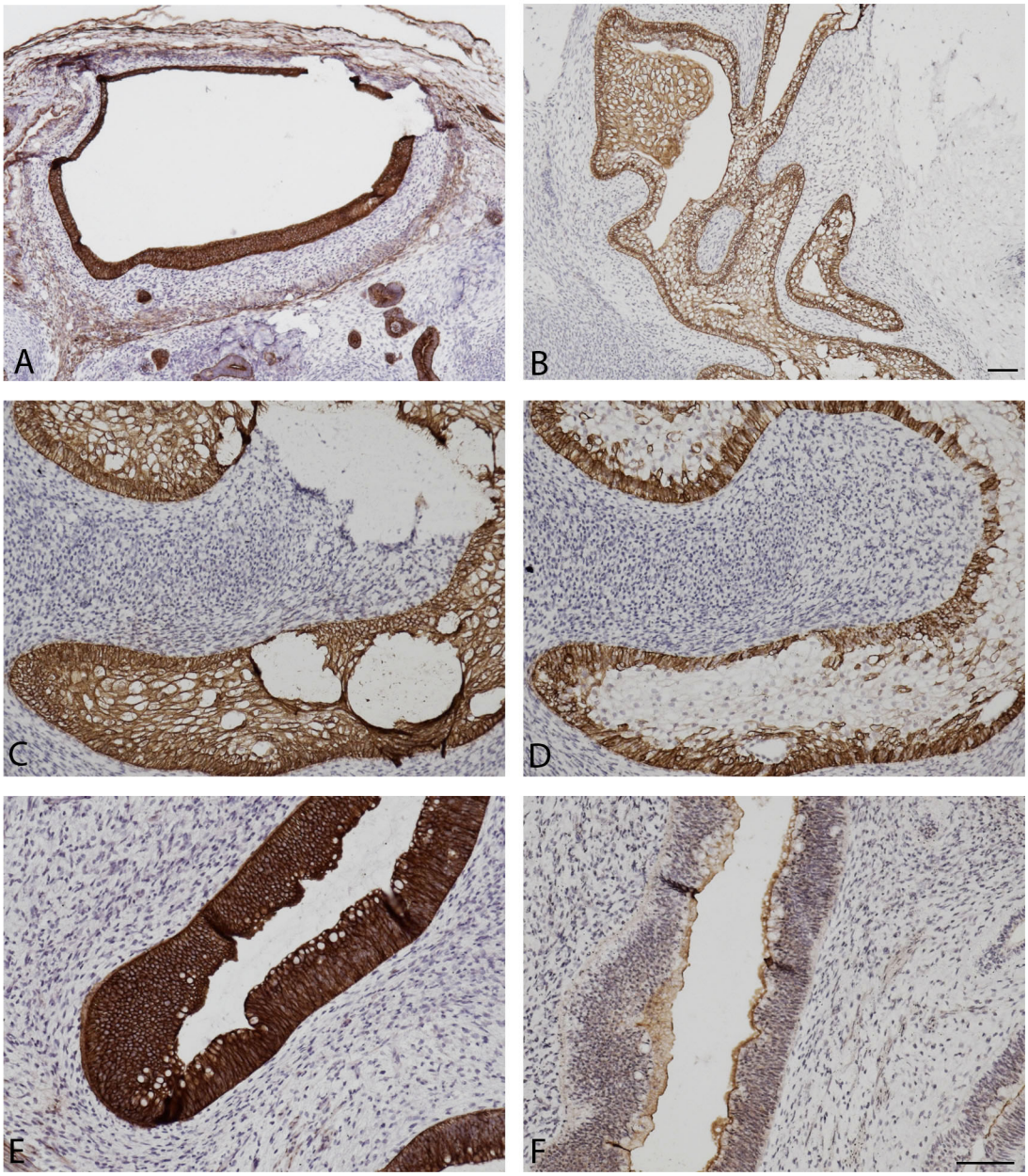

Figure 18 Immunohistochemical analysis of teratomas for epidermal-associated proteins.
A: Low magnification image showing keratin 18 positivity; **B:** Low magnification image showing keratin 5 positivity; **C and D:** Higher magnification images for keratin 5 and 14 staining, respectively; **E:** Higher magnification image showing keratin 18 positivity; **F:** Minimal involucrin positivity was also detected. Scal bars=100μm.

2.3.7 Technical discussion

To conclusively claim the true nature of immunofluorescent staining, it is crucial to demonstrate that the antibodies used are specific, selective, and reproducible in the context for which they are used. It is estimated that

there are over 180 antibody companies that produce over 350,000 commercially available antibodies against a huge collection of target proteins for the research and clinical markets (www.antibodyresource.com/onlinecomp.html). However, despite the manufacturer claims regarding the specificity of an antibody, it is not always safe to assume that a commercial antibody would only bind the desired target protein and not a range of other proteins (Couchman, 2009). Therefore, the responsibility of validating the antibody should lie within the researcher using it. Several approaches and algorithms for antibody validation have been published (Bordeaux et al., 2010, Rhodes and Trimmer, 2008, Saper and Sawchenko, 2003)

The key to proving antibody specificity is often the correct use of negative controls. As such, no primary antibody negative control during immunofluorescent analysis would provide valuable information about the antibody's specificity. However, a better negative control is a cell line or tissue that is known not to express the protein of interest. For example, fibroblast lines would serve as an effective negative control for epidermal targets, and would, therefore, be useful for validation of anti-keratin 14 antibody used in this study. In the cases when true negative lines are not available, lysates of cell lines where the target protein has been knocked down using RNAi (RNA interference) technology would provide the best negative controls. These are known as small interfering RNA (siRNA) or small harpin RNA (shRNA) knockdown controls. Similarly for a positive control, lysates from cell lines that have been transfected to overexpress protein of interest can be produced.

Western blotting is widely used to determine an antibody's specificity and is an appropriate first validation step. For this purpose, a variety of cell line lysates or tissue homogenates can be used so that both positive and negative cell lines are analyzed. The first indication that the antibody is specific for the selected target would be observing a single band at the known molecular weight for the target. For example, the expected molecular weight of the keratin 14 antibody used in this study is 55 kDa. It is worth noting, however, that the presence of multiple bands or bands at a wrong molecular weight might simply represent the same target at different post-translational modifications forms. While western blotting is a useful tool for antibody validation, an antibody demonstrating no binding on western blot may still be specific for its intended target when in its native conformation and an immunoprecipitation experiment can be the next step in determining the specificity of the antibody if the goal is for use with immunocytochemical analysis. An alternative approach for validating an antibody for immunofluorescent analysis is to titer the antibody on a tissue microarray (TMA) comprised of formalin-fixed, paraffin-embedded cell line pellets corresponding to the same cell lines used to first validate by western blotting. The usefulness of TMA as an antibody validation tool was demonstrated for HER2 testing, diagnostic of breast cancer (Kay et al., 2004). On TMA a good antibody should have the following characteristics: 1) it will stain only the cell pellets expressing the target, 2) the level of staining will decrease with increasing dilutions of the antibody, and 3) it will demonstrate an expression pattern that is consistent with biological and mechanistic data in the published literature.

2.4 DISCUSSION

In order to produce safe clinical-grade hESC lines for therapeutic purposes, propagation and maintenance of hESCs must be performed under good manufacturing practice (GMP) quality standards. In addition, all the risks of infections transmitted by animal pathogens as well as immunological reactions should be eliminated. Moreover, in order to produce sufficient numbers of differentiated cells for clinical application, culture conditions of hESCs should allow up-scaling and cost-effectiveness.

This study begun using the KCL-002 hESC line, derived and maintained under feeder-dependent conditions in the Stem Cell Biology laboratory at King's College London.

Therefore, prior to any differentiation work, two prerequisite goals had to be met.

The adaption the KCL-002 hESC line to feeder-free conditions was essential in order to overcome the following hurdles: a) the presence of immunogenic material; b) the risk of transmitting animal virus or prions; and c) difficulty with quality control of these undefined components. In addition, adaptation to enzymatical passaging in feeder-free cultures allows to significantly scale-up hESC production and enables more effective testing and application of differentiation protocols.

Secondly, once adapted to growth under feeder-free conditions, the pluripotent nature of the KCL-002 cell line had to be validated.

For feeder-free cultures several possibilities exist. Those include laminin (Genbacev et al., 2005, Xu et al., 2001, Beattie et al., 2005), fibronectin (Amit et al., 2004, Noaksson et al., 2005) and human serum coating

(Stojkovic et al., 2005). These matrices are considered to be more defined, however perhaps due to high batch-to-batch variability long-term culture of hESCs on these substrates has not been reported. Arguably the most successful commercially available matrix for long term hESCs culture is Matrigel (Sato et al., 2004, Xu et al., 2005, Xu et al., 2001). BD Matrigel™ BM Matrix is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins to include laminin (a major component), type IV collagen, heparan sulphate proteoglycans, and entactin/nidogen (Kleinman et al., 1982, Kleinman et al., 1986). Each batch of BD Matrigel has been qualified and optimized for use with mTeSR1 medium to ensure consistency and reproducibility. A comprehensive multi-centre study carried out by the International Stem Cell Initiative Consortium has assessed different defined culture systems for their ability to support hESC growth and concluded that a combination of mTeSR1 with Matrigel as coating substrate was the most effective in maintaining growth and characteristics of undifferentiated hESCs (Akopian et al., 2010). Therefore, this defined culture system was chosen for feeder-free adaptation of the KCL-002 hESC line.

The KCL-002 hESC line adapted well to growth in feeder-free conditions on Matrigel as a substrate in the presence of mTeSR1 medium. The cell line has also been adapted to enzymatic passaging using dispase, which has allowed a significant scale up in production. The results of this chapter confirm that KCL-002 hESC line grown under these conditions maintained all the characteristics of a pluripotent cell line. The expression of

pluripotency genes Nanog and Sox2 was verified by quantitative PCR and the presence of pluripotency-associated markers Oct-3/4, Sox2, Tra 1-60 and SSEA-4 was confirmed by immunocytochemistry. The KCL-002 hESC line was capable of spontaneous differentiation *in vitro* through embryoid bodies formation and primitive mesenchyme was visualized by staining with the mesodermal marker protein vimentin. Furthermore, when injected into NOD-SCID mice, KCL-002 hESCs produced teratomas confirming differentiation potential *in vivo.*

The results of this chapter demonstrate that the KCL-002 hESC line has been successfully adapted to growth in feeder-free conditions and fully characterized thus allowing to proceed with differentiation studies. However, it is worth mentioning that while Matrigel is highly effective in supporting hESCs proliferation and maintaining their pluripotent status, this basement membrane preparation is derived from EHS mouse carcinoma and therefore the culture system described here cannot be considered xeno-free (Kleinman and Martin, 2005, Kleinman et al., 1986). Hence, future work should be directed at optimizing the culture to avoid the use of any animal components.

CHAPTER 3 PROTEIN-BASED GENERATION OF HUMAN iPSCs

HYPOTHESIS

The proteins of actively proliferating hESCs in combination with epigenetic-modifying small molecules might have the capacity to induce reprogramming of adult human dermal fibroblasts (HDFs).

AIMS

1. Examine whether the transfer of proteins extracted from the KCL-002 hESC line into HDFs could achieve somatic cell reprogramming in combination with epigenetic-modifying compounds.
2. Confirm the production of iPSCs by examining expression of pluripotency-associated markers.

3.1 INTRODUCTION

3.1.1 Integration-free methods for generation of iPSCs

The discovery that various somatic cell types could be successfully reprogrammed into pluripotent stem cells offers exciting possibilities of patient-specific iPSC lines, as well as avoiding ethical issues associated with hESC application. The original iPSC induction system used retroviral vectors (Takahashi and Yamanaka, 2006), which integrate transgenes into the host genome. Integrating viral-based methods are highly efficient in producing iPSCs and are acceptable when the remaining reprogramming factors do not significantly interrupt designed assays. However, the transgene integration and alteration of the endogenous genomic organization raises a safety issue when considering medical applications. The insertion of tumourigenic genes, such as c-Myc, and activation of proto-oncogenes by long terminal repeats increase the risk of tumour formation (Okita et al., 2007, Hacein-Bey-Abina et al., 2003).

Therefore, different non-integrating approaches have been devised for somatic cell reprogramming and are summarized in Table 7. These can be divided into four main categories based on vector type for delivery of the reprogramming factors: 1) DNA-based; 2) excisable; 3) RNA-based; and 4) protein-based.

Reprogramming was achieved using non-integrating DNA-based vectors, such as adenoviruses and plasmids (Okita et al., 2008, Zhou and Freed,

2009, Si-Tayeb et al., 2010, Stadtfeld et al., 2008b), showing that iPSCs can be induced by the transient expression of reprogramming factors. However, iPSC generation by non-integrating adenoviruses and plasmids was very inefficient and estimated to be less than 0.0002%, over 1000-fold lower than that of viral transduction (Takahashi and Yamanaka, 2006). Furthermore, in a large number of iPSC clones, transgenes had been integrated into the host genome (Okita et al., 2008). Generation of iPSCs by transfection with non-integrating episomal vectors has also been reported (Yu et al., 2009).

Excisable vectors such as *piggyBac* transposon (Woltjen et al., 2009, Kaji et al., 2009), *Cre*-recombinase excisable viruses (Soldner et al., 2009) and *lox*P-flanked lentiviral vectors (Somers et al., 2010) have been used to reprogramme somatic cells. While the transgenes can be excised by inducible gene expression once reprogramming is established (Soldner et al., 2009, Woltjen et al., 2009), residual sequences and chromosomal disruptions may still result in harmful alterations that could pose clinical risks.

Several RNA-based methods have also been tested. One of the recent improvements to non-integrating methods is the application of Sendai virus (Fusaki et al., 2009). Sendai virus is a minus strand RNA virus, which replicates its genome in the cytoplasm of infected cells, and thus can stably express reprogramming factors and achieve high reprogramming efficiency. However, the established iPSCs were shown to carry the virus genome even after long-term culture. Recently, direct delivery of synthetic mRNA has been shown to generate iPSCs with high efficiency (Warren et al., 2010). However, reprogramming via modified RNAs is technically difficult,

sensitive to reagents and labour-intensive. Fast reprogramming to pluripotency using microRNAs has also been reported (Miyoshi et al., 2011).

One of the most exciting developments in the field is protein-based induction of pluripotency (Kim et al., 2009, Zhou et al., 2009). This methodology has distinct advantages over other non-integrating approaches: it avoids the risks of occasional genomic integration associated with DNA-based vectors and is much less labour intensive than current RNA-based methods. Therefore, if proven efficient, protein-based reprogramming could overcome many of the hurdles currently associated with iPSCs generation.

Type		Cell type/origin	Efficiency	Advantages	Disadvantages	References
DNA-based	**Adenoviral**	Mouse and human fibroblasts Mouse liver cells	∞0.001	Transient expression of reprogramming factors	Low efficiency	(Stadtfeld et al., 2008b, Zhou and Freed, 2009)
	Plasmid	Mouse and human fibroblasts	∞0.001	Transient expression of reprogramming factors	Low efficiency Occasional genomic integration	(Okita et al., 2008, Si-Tayeb et al., 2010)
	Episomal plasmid	Human neonatal fibroblasts	∞0.001	Cytoplasmic replication	Requires 3 individual plasmids carrying 7 factors Includes SV40 oncogene	(Yu et al., 2009)
	DNA minicircles	Human fibroblasts	∞0.001	Free of bacterial DNA Persistent high level expression	Occasional genomic integration	(Jia et al., 2010)
Excisable	**Transposon**	Human embryonic fibroblasts	∞0.1	Reasonably efficient	Labour-intensive screening of excised lines	(Kaji et al., 2009, Woltjen et al., 2009)
	Excisable lentiviral	Human fibroblasts	∞0.1-1	Reasonably efficient	Labour-intensive screening *lox*P sites retained in the genome	(Somers et al., 2010, Soldner et al., 2009)

RNA based	**Modified mRNA**	Human fibroblasts	∞1-4.4	High efficiency Evades innate antiviral response Fast and controllable	Multiple rounds of transfection required	(Warren et al., 2010)
	MicroRNA	Human and mouse adipose stromal cells and fibroblasts	∞0.1	Efficient Fast No exogenous transcription factors No risk of integration	Less efficient than other RNA based methods	(Miyoshi et al., 2011)
	Sendai virus	Human fibroblasts	∞1	High efficiency Replicates in the cytoplasm	Sequence-sensitive RNA replicase Difficult to remove replicating virus from the cells	(Fusaki et al., 2009)
Protein	**Recombinant proteins**	Human and mouse neonatal and foetal fibroblasts	∞0.001	No DNA-related complications	Low efficiency	(Kim et al., 2009, Zhou et al., 2009)
	Cell protein extracts	Mouse fibroblasts	∞0.001	No DNA-related complications	Low efficiency	(Cho et al., 2010)

Table 7 Non-integrating approaches for iPSC generation.

3.1.2 Protein-based reprogramming methods

Protein-based reprogramming methods can be subdivided into: 1) tagged recombinant proteins and 2) whole protein extracts from ESCs.

3.1.2.1 Recombinant proteins for reprogramming

A major hurdle for intracellular delivery of macromolecules such as proteins is their limited ability to cross the cellular membrane (Belting et al., 2005). The human immunodeficiency virus transactivator of transcription (HIV-TAT) protein can overcome this hurdle with a short basic segment residing at amino acid 48-60 that allows this protein to penetrate the cell membrane and activate HIV-specific genes (Frankel and Pabo, 1988, Frankel et al., 1988). It has been previously demonstrated that various proteins can be delivered into cells by conjugating them with a short peptide that mediates protein transduction, such as HIV-TAT and poly-arginine (Inoue et al., 2006, Michiue et al., 2005, Wadia and Dowdy, 2002). These naturally occurring peptides that are capable of overcoming the cell membrane barrier contain a high proportion of basic amino acids (e.g., arginine or lysine) and are known as cell penetrating proteins (CPP) (El-Sayed et al., 2009, Ziegler et al., 2005). This strategy has been exploited to create CPP-reprogramming proteins and generate iPSCs from murine embryonic fibroblasts (Zhou et al., 2009). The method involved a prolonged four-cycle treatment of murine fibroblasts with a high concentration (8mg/ml) of reprogramming factors and supplementation with VPA, and only 3 colonies per 5x10 cells were

observed after 30-35 days of culture. Subsequently, iPSCs from human fibroblasts were generated by four reprogramming proteins (Oct-3/4, Sox-2, Klf4, and c-Myc) fused with a CPP (Kim et al., 2009). In contrast to the protocol described by Zhou *et al.* (2009) the reprogramming proteins used were expressed in mammalian cells as opposed to refolded proteins after expression in *E. coli*. This difference may explain the lower efficiency of the protocol described by Zhou *et. al.* (2009), as bacterial expression systems often result in proteins that do not fold properly and are, therefore, biologically inactive. Furthermore, lack of mammalian enzymes responsible for post-translational modifications, such as glycosylation, may also result in inactive proteins. It is worth noting that the authors tested several treatment conditions until reprogramming was observed (Kim et al., 2009). In an initial series of experiments, 5×10^5 human neonatal fibroblasts were treated with combined total extracts of four HEK293 cell lines, each stably expressing one of the four human reprogramming factors (Oct4, Sox2, Klf4, and c-Myc) fused with a CPP for 16 hours, however no reprogramming was observed. Subsequently, repeated protein treatment was tested and several iPSC-like colonies were detected, but none of these colonies showed alkaline phophotase activity, suggesting incomplete reprogramming. Therefore, the authors repeated this procedure for a total of 6 cycles, and the number of iPSC-like colonies significantly increased and, as a result, two iPSC lines were established and characterized (Kim et al., 2009). Recently, the same group has reported that protein-derived iPSCs could produce fully functional dopamine neurons that could treat a rat model of Parkinson disease (Rhee et al., 2011).

3.1.2.2 Whole ES protein extracts for reprogramming

Cellular protein extracts rather than recombinant proteins have been shown to direct pluripotent stem cells differentiation into specific lineages (Hakelien et al., 2002, Qin et al., 2005), or dedifferentiation into the pluripotent state (Taranger et al., 2005). However, repeated transfer of extracts from embryonic carcinoma or ESC did not produce fully reprogrammed iPSCs in terms of the transcriptional state, *in vivo* 3 germ layer differentiation capacity, and developmental potential (Taranger et al., 2005, Rajasingh et al., 2008). Subsequently, a single transfer of ESC protein extracts, rather than repeated transfer or prolonged exposure, was shown to effectively mediate reprogramming of murine adult fibroblasts that were biologically and functionally ESCs *in vitro* and possessed *in vivo* differentiation and developmental potentials (Cho et al., 2010). The authors estimated the total amount of Oct-3/4 protein available for reprogramming in their method was 9.25ng (Cho et al., 2010), which is a substantially lower concentration than that used in reprogramming mediated by CPPs (Zhou et al., 2009). This implies that other effector molecules, which could be the downstream targets of the defined transcriptional factors, may be responsible for ES extract protein-mediated reprogramming. Extensive analysis implied that cytoplasmic proteins were equally important for induction of reprogramming as nuclear proteins (Cho et al., 2010). It has also been suggested that a certain group of ESC–derived proteins between 30-100kDa could be responsible for protein-based iPS generation (Cho et al., 2010).

3.1.3 Epigenetic modifiers to enhance iPSC generation

A global survey of DNA methylation in iPSCs revealed that pluripotent stem cells have much higher DNA methylation than somatic cell types (Doi et al., 2009, Deng et al., 2009). The upregulation of DNA methyltransferase (DNMT) 3b and 3l occurs during the reprogramming process, and might be responsible for *de novo* methylation during the production of iPSCs. Therefore, it is not surprising that epigenetic modifying drugs, such as such as 50-azacytidine and RG108, or DNMT inhibitors, can improve iPSC generation (Shi et al., 2008). Other epigenetic modifications, such as histone methylation and acetylation, are also involved in iPSC reprogramming. BIX01294 is a selective inhibitor of G9a histone methyl transferase (G9a HMTase) (Kubicek et al., 2007). G9a HMTase regulates gene expression of Oct-3/4, and BIX01294 was shown to enhance the reprogramming efficiency of neural progenitor cells (Shi et al., 2008). Addition of VPA, a HDAC inhibitor, improves reprogramming efficiency in both mouse and human fibroblasts (Huangfu et al., 2008b, Huangfu et al., 2008a). Other HDAC inhibitors, suberoylanilide hydroxamic acid, trichostatin A and sodium butyrate, can also greatly increase iPSC generation (Huangfu et al., 2008a, Ware et al., 2009, Mali et al., 2010).

3.1.4 Inhibition of p53 pathway to increase reprogramming efficiency

The expression of pluripotency marker genes is often found in immature tumour cells. For example, breast cancer cells show elevated levels of Oct-

3/4 and Nanog (Ezeh et al., 2005). In fact, both immature tumour cells and iPSCs are quite similar in their origin and derived from somatic cell types, which acquire differentiation potential to transform into different cell types. The p53 pathway reduces cancer initiation by inducing apoptosis or cell cycle arrest in response to a variety of stress signals, including over-expressed oncogenes such as c-Myc, one of the major transcription factors for reprogramming (Nakagawa et al., 2008). Another important reprogramming factor, Klf4, can either activate or antagonize p53, in a context-dependant manner (Rowland et al., 2005). Several studies have examined the effect of p53 inhibition on the reprogramming efficiency (Hong et al., 2009, Kanatsu-Shinohara et al., 2004, Kawamura et al., 2009, Zhao et al., 2008). Germ cells were shown to undergo spontaneous reprogramming in the absence of p53 (Kanatsu-Shinohara et al., 2004) and p53 siRNA expression increased iPSC formation (Zhao et al., 2008). Accordingly, reprogramming somatic cells to iPSCs was shown to be associated with activation of the p53 pathway, which serves as a barrier to reprogramming (Kawamura et al., 2009). The transduction of p53-null MEFs with the four reprogramming factors revealed marked enhancement of iPSC generation (Hong et al., 2009). Furthermore, improved reprogramming efficiency was also reported when wild-type adult HDFs were transduced with a dominant-negative form of p53 or its short hairpin RNA (Hong et al., 2009).

These collective data imply that the efficiency of somatic cell reprogramming is reduced through oncogene-mediated activation of the p53 pathway, and that direct chemical inhibition of the apoptotic cascade may

provide a useful tool for enhancing reprogramming efficiency. However, permanent p53 suppression may lead to genomic instability and thereby increase the probability of malignant transformation; therefore, continuous suppression should be avoided (Marion et al., 2009a). Rather, transient inhibition using chemical antagonists, such as pifithrin (PFT-α), that avoid genetic disruption could be employed (Komarov et al., 1999). Cyclic PFT-α (cPFT-α) was found to be one log more potent than PFT-α for p53 inactivation (Pietrancosta et al., 2005), and therefore, was used in my studies.

For my set of experiments, it was hypothesized that protein extracts of actively proliferating KCL-002 hESCs, in combination with epigenetic-modifying small molecules and p53 inhibitors, can induce generation of iPSCs from human somatic cells, namely HDFs.

3.2 METHODS

3.2.1 hESC proteins extraction

Prior to protein extraction, the KCL-002 hESC line was cultured under feeder-free conditions as described in section 2.2.5. Proteins were extracted using Cayman's protein extraction kit according to manufacturer's instructions (Cayman, USA; distributed by Cambridge Bioscience Ltd., Cambridge, UK). Undifferentiated hESCs were collected with dispase (Sigma) and centrifuged at 300g for 5min. The resulting cell pellet was resuspended to a single cell suspension in 1ml mTESR medium. Following

cell counting, $1x10^7$ cells were transferred into a pre-chilled 15ml tube and centrifuged at 300g for 5min at 4°C. After that, the supernatant was discarded and the cell pellet was resuspended in 5ml ice-cold PBS solution containing phosphatase inhibitors and centrifuged again at 300g for 5min at 4°C. The phosphatase inhibitors limit events controlled by dephosphorylation, including, but not limited to, transcription factor activation, movement of proteins in and out of the nucleus, proteolysis, and new protein expression. Once the supernatant was discarded, 500µl ice-cold 1x Hypotonic buffer was added to the cell pellet, mixed gently by pipetting, and transferred to a pre-chilled 1.5ml microcentrifuge tube. The cells were then placed on ice for 15min to allow the cells to swell and increase membrane fragility. After that, 100µl of 10% Nonidet P-40 (nonionic, non-denaturating detergent) reagent was added and mixed gently by pipetting. Addition of detergent breaks the cell membrane allowing access to cytoplasmic proteins fraction while maintaining the integrity of the nuclear membrane. Pulse spin centrifugation for 30s at 4°C was performed in a microcentrifuge. The resulting supernatant contained the cytoplasmic fraction of hESC proteins. A small (1µl) amount was kept for quantification and the rest was transferred to a new 1.5ml microcentrifuge tube and stored at -80°C until transfer into HDFs. The pellet was resuspended in 50µl ice-cold 1x Complete Nuclear Extraction buffer containing 10mM dithiothereitol as well as protease and phosphatase inhibitors, vortexed for 15s at the highest setting, then gently rocked on ice for 15min using a shaking platform, vortexed for a further 30s at the highest setting and then gently rocked for an additional 15min. After centrifugation at 14,000g for 10min at

4°C, the resulting supernatant contained the nuclear hESC proteins fraction. A small (1µl) amount was kept for quantification and the rest was stored at -80°C until transfer to HDFs.

3.2.2 Protein quantification

Protein concentration was measured by the Bicinchoninic Acid (BCA) protein assay kit (Thermo scientific). This assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by BCA (Smith et al., 1985). The first step is the chelation of copper with protein in an alkaline environment to form a light blue complex. In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a coloured chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate.

In the second step of the colour development reaction, BCA reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-coloured reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562nm with increasing protein concentrations. The purple colour can be measured at any wavelength between 550nm and 570nm with minimal (less than 10%) loss of signal. The BCA reagent is approximately 100 times more sensitive (lower limit of detection) than the pale blue colour of the first reaction.

Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as BSA. A series of dilutions of known concentration were prepared using the same diluents as for the respective sample. Final BSA concentrations (µg/mL) in standard were as follows: 2000, 1500, 1000, 750, 500, 250, 125 and 25. Since the cytoplasmic proteins fraction contains a small amount of detergent, which can interfere with accurate protein quantification, 1x Hypotonic buffer without Nonidet P-40 was used as the blank and for diluting the cytoplasmic fraction 1:25 prior to protein quantification. For nuclear samples, 1x Extraction buffer was used as the blank and for diluting samples.

A total volume of 25µl of diluted cytoplasmic and nuclear fractions was added to a microplate well in duplicate. The total volume of the BCA working reagent required was calculated using the following formula: (4 standards + 2 samples) × (2 replicates) × (200µl) = 2.4ml. The BCA working reagent was prepared by mixing BCA Reagent A with BCA Reagent B at a 50:1 ratio. BCA Reagent A consists of sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide; BCA Reagent B contains 4% cupric sulphate. After 200µl of the BCA working reagent was added to each well, the microplate was rocked thoroughly on a plate shaker for 30s. The plate was covered and incubated at 37°C for 30min. Once the plate was cooled to room temperature, the absorbance was measured at 562nm on an ELISA plate reader. The average 562nm absorbance measurement of the blank replicates was subtracted

from the 562nm measurements of all individual standards and cytoplasmic and nuclear fraction replicates, and a standard curve was prepared by plotting the average blank-corrected 562nm measurement for each BSA standard vs. its concentration in µg/ml. The standard curve was then used to determine the protein concentration of cytoplasmic and nuclear fraction samples. A concentration of 35mg/ml of both cytoplasmic and nuclear proteins was used for protein-based reprogramming of HDFs.

3.2.3 hESC protein extracts transfer

A simplified scheme for protein-based reprogramming of HDFs is outlined in Figure 19. Following the protein transfer HDFs were cultured with or without supplementation with the small-molecule compounds. In addition, when small molecules were added to the culture medium, three working concentration of cPFT-α were tested. Therefore, a total of 4 experimental groups were investigated.

HDFs were washed in PBS and harvested with 0.1mM trypsin/1mM EDTA solution. For each condition tested, a total of $8x10^6$ HDFs were collected by centrifugation at 500g for 10min at 4°C, and 10^6 cells were resuspended in separate 1.5ml tubes containing 977µl cold Ca2+- and Mg2+-free Hank's balanced salt solution (HBSS; Invitrogen) in 1.5ml tubes (8 technical replicates). The tubes were placed in a water bath at 37°C for 2min, and 23µl of streptolysin O (SLO; Sigma-Aldrich) (100µg/ml stock diluted 1:10 in cold HBSS) was added to achieve a final SLO concentration of 230ng/ml.

The pore-forming toxin SLO is frequently used to reversibly permeabilize adherent and nonadherent cells, allowing delivery of molecules of up to 100kDa mass to the cytoplasm (Walev et al., 2001). Samples were incubated horizontally in a water bath for 50min at 37°C with occasional agitation and placed on ice for 5min, and the cells were then collected by centrifugation at 600g for 3min at 4°C. After permeabilization, cells were suspended at 2000 cells/µl in 200µl of hESC-derived extract containing an ATP-generating system (1mM each nucleotide triphosphate, 10mM phosphocreatine, and 25µg/ml creatine kinase; all Sigma) and incubated for 1 hour at 37°C in a water bath with occasional agitation. To reseal plasma membranes, the cell suspension was diluted with 1ml mTESR medium containing 2mM $CaCl_2$, and incubated for 2 hours at 37°C. After that time, the cell suspension was placed in 15ml tubes and washed in 10ml PBS and centrifuged at 600g for 10min at 4°C. Cells were then resuspended in mTESR medium and seeded on Matrigel (BD) coated 6 well culture plates (Appleton Woods) at a density of 10^6 cells per well. The medium was changed daily with or without small-molecule compounds. As discussed previously, O_2 tension is an important aspect for hESCs maintenance and differentiation (Ezashi et al., 2005), and generation of iPSCs performed in hypoxic conditions (5% O_2) shows up to four-fold increase in the reprogramming efficiency in both mouse and human fibroblasts (Yoshida et al., 2009). Therefore, following protein transfer, HDFs were cultured under hypoxic conditions.

The experiment was repeated twice with HDFs thawed and recovered from different vials (n=2 biological replicates).

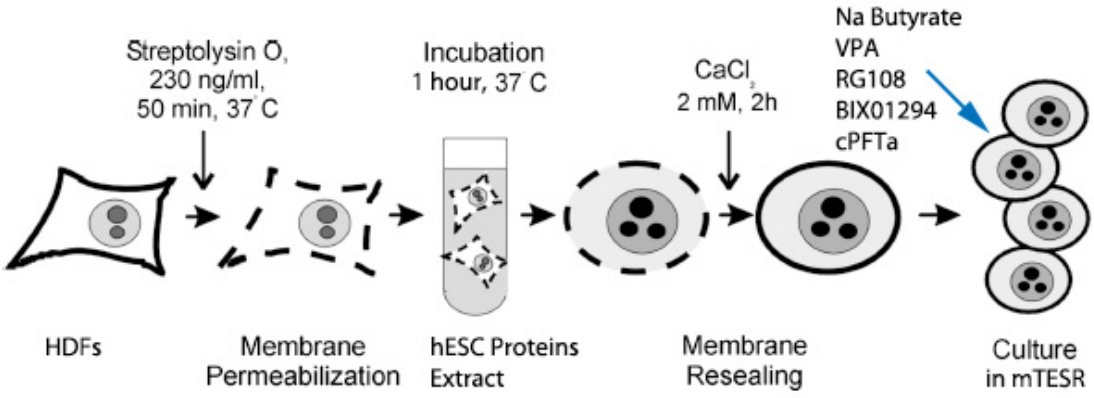

Figure 19 A schematic representation of the protein-based reprogramming protocol.
HDFs are reversibly permeabilized by SLO treatment and incubated with hESC proteins extract for 1 hour. At the end of the incubation time, the cell membrane is resealed by $CaCl_2$, and the cells are cultured in mTESR medium with or without small-molecule compounds (blue arrow). Modified from (Walev et al., 2001)

3.2.4 Preparation of small molecules to enhance protein-based reprogramming

In general, small-molecule compounds were dissolved to 1/1000 concentration, stored at -20°C, and added fresh to the mTESR media to the working concentration desired before every media change. Three working concentrations of cPFT-α (Tocris, Bristol, UK) were tested: 10μM, 1μM and 100nM. According to previously published optimal conditions (Huangfu et al., 2008a), following protein transfer, HDFs were treated with 1mM VPA (Stemgent, supplied by Miltenyi, Bergisch Gladbach, Germany) for 10 days. BIX01294 (Stemgent) was used at a 1μM working concentration, which has previously been shown to effectively improve reprogramming (Shi et al., 2008). RG108 (Stemgent) was used at a 5μM working concentration. Sodium butyrate (Stemgent) was dissolved to a concentration of 1M, and stored at -

20°C in glass vials and a working concentration of 0.25mM was used (Mali et al., 2010). The small molecules used are summarized in Table 8.

Small-molecules	Function	Concentration used	References
cPFT-α	p53-pathway inhibitor	10μM,/1μM/100nM	Kawamura *et al.*, 2009
VPA	HDAC inhibitor	1mM	Huangfu *et al.*, 2008a
BIX01294	G9a HMTase inhibitor	1μM	Shi *et al.*, 2008
RG108	DNMT inhibitor	5μM	Shi *et al.*, 2008
Sodium butyrate	HDAC inhibitor	0.25mM	Mali *et al.*, 2010

Table 8 Summary of compounds used in to enhance iPSC generation.

3.2.5 Immunocytochemistry

When small clumps of cells appeared in cultures of HDFs that had received hESCs proteins, they were transferred to a Matrigel-coated tissue culture 4 well plate and cultured for a further 14 days in mTESR medium. The iPS-like colonies were first partly subcultured onto a new Matrigel-coated tissue culture 4 well plate, and then fixed in 4% PFA and stained for pluripotency-associated markers, as described in section 2.2.7. The following antibodies were tested: mouse anti-SSEA-4 and rabbit anti-Nanog (Abcam).

3.3 RESULTS

The concentration of hESC-derived proteins was 2000μg/μl and 7200μg/μl for nuclear and cytoplasmic fractions, respectively, as measured by BSA assay. After 7 days of culture, some small clumps of cells were observed

when epigenetic-modifiers were used (Figure 20A and B); no clumps were observed when supplementation with small-molecule compounds was omitted. The number of clumps varied depending on the concentration of cPFT-α used: 0-2 in 10μM, 4-6 in 1μM and 1-3 in 100nM. This suggested that the optimal concentration of cPFT-α was 1μM. All clumps were manually picked and transferred onto new Matrigel coated dishes in mTESR in an attempt to obtain iPSC colonies. Out of total 16 clumps recovered, only 2 grew into colonies with an iPS-like morphology, as evaluated under phase-contrast microscopy: colonies appeared to be tightly packed with a defined border; individual cells were uniform with phase bright edges, a high nuclear/cytoplasmic ratio, and prominent nucleoli (Figure 20C). Both colonies were subcultured onto a new Matrigel coated dishes before being fixed and processed for immunocytochemistry. Due to a low yield of putative iPS-like colonies only two pluripotency-associated markers could be tested for by immunocytochemistry, namely SSEA-4 and Nanog. The colonies stained positive for SSEA-4 (Figure 20D). However, Nanog expression was not detected detected (Figure 20E). Following subculture, excessive apoptosis was observed and the colonies could not be further expanded.

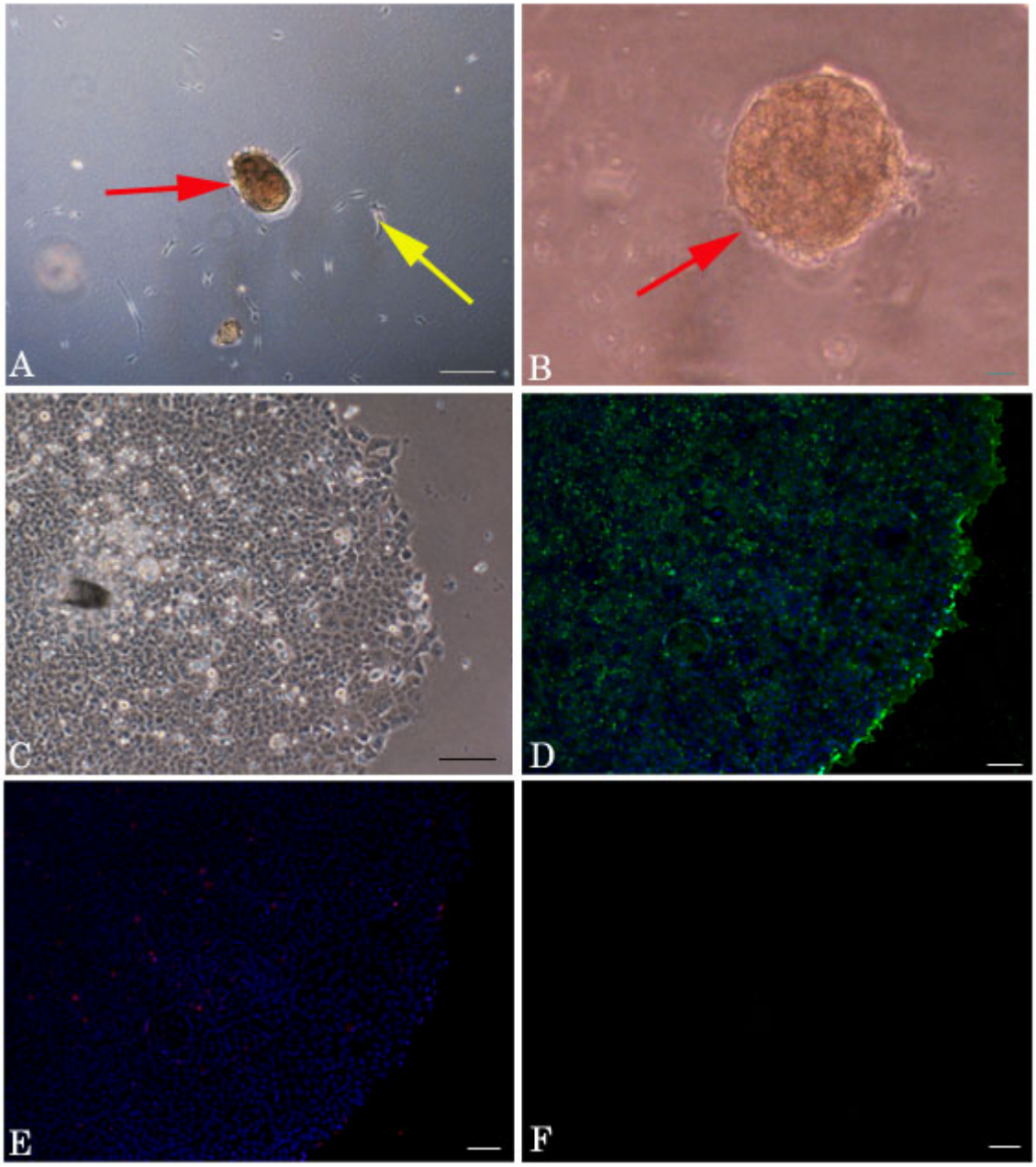

Figure 20 Morphological appearance and expression of pluripotency markers of cells following transfer of hESC-derived proteins.
A, B: Small clumps of cells appear after approximately 7 days of culture following hESC-proteins transfer (red arrow). Some cells with typical fibroblast morphology can also be seen (yellow arrow); **C:** Colonies resembling iPSCs appear after subsequent 14 days of culture; **D:** These colonies only stain positive for the pluripotency marker SSEA-4; **E:** However, the putative iPSC colonies are negative for Oct-3/4. **F:** No primary antibody negative control. Scale bars=50μm.

3.4 DISCUSSION

Protein-based reprogramming of adult somatic cells offers prospects of a completely nucleic acid-free methodology, which, therefore, avoids any risks of genomic integration and associated mutagenesis. It is also relatively simpler technically than current RNA-based methods. Therefore, in this set of experiments I sought to investigate whether HDFs could be successfully reprogrammed using whole protein extracts from the KCL-OO2 hESC line. Such protein-derived iPSCs could potentially be used for subsequent derivation of epidermal cell lineages. Furthermore, patient specific iPSCs could be generated for tailored therapy of RDEB when revertant mosaicism is detected (Almaani et al., 2010, Pasmooij et al., 2010, van den Akker et al., 2011). Unfortunately, despite a high concentration of hESC proteins and application of epigenetic-modifying factors, protein-based reprogramming of HDFs did not yield any true iPSC colonies. Considering, the low efficiency of previously reported protein-based generation of iPSCs from mouse fibroblasts (Cho et al., 2010), it is very likely that comprehensive optimization would be required to yield positive results with human fibroblasts, which would have extended well beyond the time frame of this PhD study.

Following the transfer of hESC-derived protein extracts into HDFs and application of small-molecule compounds to enhance reprogramming, only 2 iPS-like colonies were observed after careful culture. These colonies were composed of tightly packed homogenous cells with a defined border. The

individual cells had phase bright edges, a high nuclear/cytoplasmic ratio, and prominent nucleoli. Despite their morphological similarities to pluripotent stem cells, these colonies tested negative for the expression of pluripotency-associated transcription factors Oct-3/4 and Nanog, although SSEA-4-positivity was observed. This observation indicated that full reprogramming to iPSC state was not achieved.

Previously, it was shown that the ectopic expression of defined factors frequently results in partially reprogrammed "intermediate" cell populations (Chan et al., 2009, Mikkelsen et al., 2008) suggesting that transfer of proteins into some cells might be insufficient for reprogramming up to pluripotency or protein-based reprogramming approach might be also a stochastic rather than deterministic process (Hanna et al., 2009). When the relationship between cell number and reprogramming efficiency was examined using the secondary induction system, the authors claimed that reprogramming is a continuous stochastic process dependent on such events as the initial cell condition, microenvironment, variation in gene expression and epigenetic modification (Hanna et al., 2009). The authors of that study argued that all target cells would eventually give rise to iPSCs after extended cultivation. For this reason, the colonies obtained in this set of experiments were further subcultured in an attempt to assess where full pluripotency status can eventually be achieved. However, the cells underwent apoptosis and expansion could not be attained.

In the original study, on which this set of experiments was based, 5-10 colonies were obtained from $1x10^6$ primary cardiac fibroblasts (Cho et al.,

2010). This is comparable to the amount of clumps observed when epigenetic-modifying small molecules and 1μM cPFT-α were added to culture medium. Consequently, the authors have reported that only 2 iPSC lines were established (Cho et al., 2010).

An important consideration is that marked differences in protein expression between different ESC lines can lead to significant differences in reprogramming efficiency. Cho *et al.* (2010) achieved successful reprogramming when the C57 ESC line was used for protein extraction. However, when a different ESC line was used as protein donor no reprogramming was observed. Therefore, it is plausible that the specific protein expression in the KCL-002 hESC line was insufficient for reprogramming of HDFs.

Overall, the establishment of human iPS-like colonies using protein-based methods takes about 8 weeks (Kim et al., 2009), approximately double that seen with viral transduction (Park et al., 2008b, Takahashi et al., 2007, Yu et al., 2007). Furthermore, at present, the efficiency of iPS generation using protein-based methods is only about 0.001%, which is significantly lower compared to virus-based protocols (cca 0.01%) (Park et al., 2008b, Takahashi et al., 2007, Yu et al., 2007). Furthermore, the reprogramming efficiency of human fibroblasts is approximately 10-fold lower than that of mouse fibroblasts when retroviral vectors, the most efficient tool for iPSCs generation, are used (Takahashi et al., 2007, Takahashi and Yamanaka, 2006). Considering that the protein-based method has a very low efficiency even in mouse fibroblasts (Cho et al., 2010), there was a strong possibility

that the generation of human iPSCs also might not be successful using the same method. It is unclear whether an iPSC generation method with such a low efficiency would offer any therapeutic value for patients with RDEB. In order to gain some clinical potential for protein-based reprogramming of human fibroblasts, extensive revisions and modifications would presumably be required, which could not be accommodated within the time frame of this PhD study. Therefore, no further work was undertaken in this field for now.

CHAPTER 4 ORGANOTYPIC CULTURES TO PROMOTE DIFFERENTIATION OF hESCs TOWARDS EPIDERMAL LINEAGES.

HYPOTHESIS:

Undifferentiated hESCs exposed to a complex, tissue-specific microenvironments such as de-epidermized dermis of skin, in combination with appropriate tissue culture conditions, might be induced to undergo efficient epidermal differentiation.

AIMS:

1. Devise organotypic culture conditions which would enable hESCs to differentiate towards keratinocyte lineages and form epidermis, specifically stratified keratinizing epithelium.
2. Evaluate the efficiency of differentiation by morphology and markers of differentiation.

4.1 INTRODUCTION

4.1.1 Decellularized matrices for tissue engineering and regenerative medicine

Functional replacement of whole organs and complex tissues in regenerative medicine and tissue engineering relies on the use of natural readily available ECM, obtained by the decellularisation of allogeneic or xenogeneic whole organs or tissues. These decellularized matrices serve as an inductive three-dimensional biological template around which a functional tissue can be rebuilt by either recruiting endogenous cells or assembling exogenously provided replacement cells with the appropriate spatial distribution and functional maturation of these cells (Chen et al., 1999, Dahl et al., 2003, Badylak, 2007). Successful proof of principle of the potential therapeutic approaches has been shown for such organs and complex tissues as liver (Uygun et al., 2010, Soto-Gutierrez et al., 2011), heart (Ott et al., 2008), lung (Ott et al., 2010, Petersen et al., 2010, Daly et al., 2012), oesophagus (Badylak et al., 2011), and skeletal muscle (Mase et al., 2010). One of the most successful applications of this type of therapeutic strategy was achieved when a decellularized human trachea in a bioreactor was reconstituted with cultured autologous respiratory epithelial cells and autologous chondrocytes of bone marrow-derived mesenchymal stromal cell origin, and the resultant graft was used to replace a terminally diseased left main bronchus (Macchiarini et al., 2008). Since then the procedure has been further improved by shortening the time required for decellularisation of the

trachea and by use of the recipient's body as a bioreactor (Laurance, 2010). Subsequently, this approach was used in nine paediatric and adult patients with benign and malignant diseases. It is important to note, however, that in three patients a partial collapse of the scaffolds was observed, and a number of improvements in decellularisation, recellularisation, biomechanical stabilisation, and implantation approaches for tracheal grafts are now under investigation (Curcio et al., 2010, Baiguera et al., 2012).

4.1.2 Organotypic cultures as *in vitro* model of the reconstruction of skin

In organotypic cultures, keratinocytes are cultured at an air-liquid interface on a dermal equivalent such as de-epidermized or devitalized dermis (DED) (Régnier and Darmon, 1991, Gibbs et al., 1997, Ponec et al., 1997).

The critical point of harvesting the ECM from an organ or tissue is a complete removal of cells from their integrin-bound anchors and intercellular adhesion complexes while maintaining the structure, composition, and ligand background of the native matrix. The first method of recombining epidermal and dermal elements for keratinocyte culture came from culturing human skin explants on flaps on dead pig skin (Freeman et al., 1976). Subsequently, a more physiological system, namely DED, was developed (Prunieras et al., 1979). DED is a dead acellular dermal component of the skin that is still covered by mostly intact lamina densa of BM (Régnier M. et al., 1981), however, some antigens such as 180-

kDa bullous pemphigoid were found to be missing indicating some constitutive differences from the *in vivo* state (Woodley et al., 1982).

The cultivation of epidermal keratinocytes on DED has been shown *in vitro* to express all the morphologic markers of epithelial differentiation (Prunieras et al., 1983). Moreover, a positive influence from fibroblasts on keratinocyte proliferation has been known for a long time (Rheinwald and Green, 1975), and, accordingly, pre-seeding DED with both fibroblasts and keratinocytes exhibit very good epidermal morphology and production of ECM components *in vitro* (el-Ghalbzouri et al., 2002, Marinkovich et al., 1993, Smola et al., 1998). Co-culture of keratinocytes or keratinocyte progenitors with dermal fibroblasts was shown to replicate the physiological conditions *in situ* and encourage keratinocyte terminal differentiation and increased synthesis of ECM and other BM components. Furthermore, such co-culture systems enable extensive morphogenesis and subsequent formation of epidermis-like structures (Ralston et al., 1999, Krejci et al., 1991). The positive effects of fibroblasts on keratinocyte differentiation are consistent with the inductive role of the dermis during embryonic development (Sengel, 1976).

Therefore, organotypic cultures represent *in vitro* models of epidermogenesis that have been widely used to study cellular interactions, gene expression and wound healing (Parenteau et al., 1991, Fuchs, 1993, Smola et al., 1993, Garlick and Taichman, 1994, Boyce, 1996). In addition, such cultures provide a unique tool for pharmacological and toxicology studies *in vitro* (Gay et al., 1992, Ponec and Kempenaar, 1995).

4.1.3 Organotypic cultures for skin grafting

DED has been extensively studied as a potential therapeutic dermal equivalent to improve keratinocyte grafting in wounds (Heck et al., 1985, Krejci et al., 1991, Cuono et al., 1986, McKay et al., 1994, Ghosh et al., 1997). Keratinocytes from a small skin biopsy can be expanded to generate large, multilayered epithelial sheet grafts within 3–4 weeks in culture (Green et al., 1979), but on application to full thickness wounds, these grafts are unstable and fragile due to a flat DEJ and perhaps lack of mechanical support, tend to blister, and can lead to subsequent scarring (Woodley et al., 1990). This suggests that the lack of dermal component renders poor take rates of epidermal cover (Cuono et al., 1986, Navsaria et al., 1994). Consequently, DED has been used successfully for the resurfacing of full-thickness wounds (Krejci et al., 1991, Gustafson and Kratz, 1999) and also cultured in combination with fibroblasts and/or keratinocytes (Régnier et al., 1984, McKay et al., 1994, Ghosh et al., 1997). Accordingly, organotypic cultures are frequently used in skin tissue engineering, where there is a requirement for both epidermal and dermal cellular components within different sections of the same matrix scaffold (Parenteau et al., 1992, Horch et al., 2005, Huang et al., 2003). In addition, unseeded human DED has been successfully used for ventral hernia repair (Brewer et al., 2011).

4.1.4 Organotypic culture to induce epidermal differentiation of hESCs

Organotypic culture systems offer an attractive approach to direct cellular differentiation *in vitro*, and various modifications of this technique are

available (Parenteau et al., 1992, Horch et al., 2005, Margulis et al., 2005, Supp and Boyce, 2005). Previously, the utility of whole acellular lung for the development of engineered lung tissue from murine ESCs was demonstrated (Cortiella et al., 2010). It was found that acellular lung allowed for better retention of cells with more differentiation of mouse ESCs into epithelial and endothelial lineages than commercially available matrices. The authors observed some organization of differentiating ESCs into three-dimensional structures reminiscent of complex tissues, as well as expression of immature lung epithelial cell and pneumocyte markers (Cortiella et al., 2010). In another study, rat renal ECM was successfully seeded with mouse ESCs infused through the renal artery and the ureter, and proliferation and cell-specific differentiation of the stem cells within the glomerular, vascular, and tubular compartments was observed (Ross et al., 2009). These studies proved proof-of-concept that an appropriate three-dimensional tissue scaffold can successfully direct ESCs towards a required cell lineage.

Previous mouse studies utilized DED to induce epidermal differentiation from murine hESCs though embryoid body formation (Bagutti et al., 2001). The aim of that study was to investigate the reasons for failure of ß1-null mouse ESCS to undergo epidermal differentiation *in vitro*, while maintaining an ability to produce keratinocytes through subcutaneous teratoma formation in syngeneic mice or within the epidermis of chimeric wild-type/ß1-null mice (Bagutti et al., 1996). Prior to seeding onto the BM of the DED, wild-type and ß1-null murine ESCs were allowed to differentiate into embryoid bodies (Bagutti et al., 2001). After 16-26 days in culture, a small number (4.6±2 per section) of cysts resembling stratified squamous

epithelia were detected on the DEDs populated with wild-type murine ESCs, but not with β1-null cells. Interestingly, the authors have shown that co-culture with epidermal keratinocytes had no effect on the ability of ESCs to undergo epidermal differentiation. However, consistent with the inductive role of the dermis during embryonic development (Sengel, 1976) and positive effects on normal human keratinocyte proliferation, the incorporation of human dermal fibroblasts significantly increased (from 4.6±2 to 15.6±6 per section) the number of epidermal cysts formed by wild-type ESCs . Furthermore, small clumps of keratin 14-positive cells were also observed in β1-null ESC cultures. Because there was no direct contact between the two cell types, growth factors secreted by fibroblasts were suggested to be responsible for the enhanced differentiation. Consequently, it has been proposed that the inability of β1-null ESCs to undergo epidermal differentiation *in vitro* is due to a reduced sensitivity to such soluble factors.

With respect to assessing the ability of organotypic cultures to direct epidermal differentiation of hESCs only a very limited number of studies has been conducted (Hewitt et al., 2009). Hewitt et al. (2009) cultured a mixture of hESC-derived keratin 18-positive epithelial precursors and mesenchymal cells at an air-liquid interface. Employing this method requires that the cultures are fed from underneath and are exposed to the oxygenated air (Prunieras et al., 1983). Using this approach, the authors successfully detected expression of several keratins and BM proteins (Hewitt et al., 2009). However, neither keratin 14 nor p63 expression was detected, suggesting that these progenitors have not acquired the basal epidermal phenotype. In another study, dermal constructs using rat tail

collagen I demonstrated the ability of hESC-derived keratinocytes to terminally differentiate and undergo stratification and cornification (Metallo et al., 2010b). However, the profile and strength of terminal differentiation markers differed to that of *in vivo* samples. The study showed that the cells continued to proliferate and maintained p63 expression in the basal layers after 3 weeks at an air-liquid interface; however, the basal compartment was much less defined compared to human skin or primary foreskin keratinocyte-containing tissues.

To date, no studies utilizing DED as an inducer of epidermal differentiation of hESCs have been reported. Since the three-dimensional tissue architecture in organotypic co-cultures enables human keratinocytes to recapitulate their tissue-specific differentiation program *in vitro*, it is plausible that when undifferentiated hESCs are subjected to such conditions, which mimic the natural microenvironment and provide inductive cues from the underlying mesenchymal component, efficient epidermal differentiation could be promoted.

4.2 METHODS

4.2.1 Human Embryonic Stem Cell Culture

Undifferentiated KCL-002 hESCs were cultured under defined feeder-free conditions on Matrigel in mTESR1 medium (Stem Cell Technologies) as described in section 2.2.5.

4.2.2 DED preparation

The Euro Skin Bank in Holland (www.euroskinbank.org) provided the glycerol-preserved cadaveric skin that was used for DED preparation. The preserved skin was provided in a sealed and labelled clear plastic bottle and was removed from its original packaging and washed six times with PBS. It was then placed in a 500ml sterilized bottle containing 300ml of PBS and antibiotic mix (600 units/mL penicillin-G, 600mg/mL streptomycin sulphate, and 250mg/mL gentamycin sulphate; all from Sigma) and stored in an incubator at 37°C for 10 days. This process allows for the separation of the dermis from the epidermis (Ghosh et al., 1997). After day 10, the storage container was removed from the incubator and the skin was removed using sterile forceps. The epidermis was peeled away easily at this point and any remaining epidermis and cells was washed away as the skin was washed with PBS three times. The DED was then equilibrated in a mixture of DMEM (Invitrogen) and 10% foetal calf serum (FCS) at 37°C in 10% CO2 for 2 hours. The large piece of skin was then cut with a sterile disposable surgical knife into 1.5x1.5cm squares ready for use.

4.2.3 Experimental groups

Four experimental conditions were tested; these are shown in Figure 21. DED was inoculated with hESCs alone, or in combination with normal human keratinocytes (NHKs). The third experimental group involved addition of laminin-332 alpha3 sequence to DEDs to aid cell adhesion. ATRA was added to the differentiation medium in the fourth experimental

groups. All experimental groups were done with or without addition of HDFs to the reticular side of the DED.

4.2.4 Seeding DEDs with cells

The 1.5cm² DED pieces were placed in six-well cell culture plates and then 1cm-diameter stainless steel rings were placed on top of each DED.

For the fibroblast-containing experimental groups, HDFs were seeded as follows. HDFs were collected from the flask using 0.05% trypsin in 0.02% EDTA (Invitrogen), counted using a haemocytometer, and centrifuged at 400g for 5min. The cells were resuspended in DMEM with 10% FBS and were seeded into the rings on the reticular dermal surface of the DED at a density of $2x10^6$ cells per DED in 1ml volume. Medium was also added to the area surrounding the DEDs. Following 24 hours incubation, the rings were removed and DEDs were inverted to orient the papillary dermal surfaces on top before the rings were replaced on the DEDs as before.

To seed hESCs, undifferentiated colonies were enzymatically passaged using dispase, centrifuged at 300g for 5mins and resuspended in mTESR1 in a single-cell suspension. The cells were counted and $2x10^6$ cells per DED were added to the papillary side. When co-culture of hESCs with NHKs was employed, $1x10^6$ of each cell type was seeded per DED. NHKs were grown in Defined Keratinocyte Serum-Free Medium (DKSFM; Invitrogen) and collected by incubation with TrypLE™ Express (Invitrogen) for 5min at 37°C. For experimental groups containing laminin-332 α3 sequence, human

recombinant laminin peptide sequence PPFLMLLKGSTR was added to the reticular side of DEDs at a concentration 70µl/ml. The laminin peptide was purchased from GenScript Corporation (Piscataway, USA). Peptide sequence was synthesized by manual solid phase peptide synthesis on 2-chlorotrityl chloride resin using standard procedures (Chen and White, 2000). Briefly, the pentafluorophenyl esters of 9-fluorenyl methoxy carbonyl (Fmoc) amino acids were used in the process along with 1-hydroxybenzotriazole. The peptides were cleaved from the resin with a trifluoroacetic acid/thioanisole/ethanedithiol/ anisole (9:0.5:0.3:0.2) mixture and precipitated with cold diethyl ether. The purity and composition of the peptide was determined using high-performance liquid chromatography and amino acid analysis, respectively. The purified peptide was identified using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and the purity of peptide was found to be >99%.

Once seeded onto DED, hESCs were allowed to attach and adapt in mTESR1 for 2 days.

4.2.5 Differentiation protocol

After 2 days, the cultures were raised to the air-liquid interface by placing DEDs on stainless steel grids. The medium containing 25ng/ml BMP-4 (R&D Systems) was added to all experimental groups. In addition, one experimental group also had 1µM ATRA (Sigma) applied to the differentiation medium. The medium was changed every 2 days for 1 week (days 3-9 inclusively). At day 10, the medium in all experimental groups was

changed to DKSFM in order to propagate the potential epidermal progenitors. DKSFM was changed every 2 days for a further 2 weeks (days 10-24 inclusively). Each experimental group was done in duplicate (technical replicates). The experiment was independently repeated three times with different batches of DEDs (n=3 biological replicates). The differentiation protocol is outlined in Figure 21.

DED with HDFs only, and hESCs seeded on Matrigel directly onto a six-well cell culture plate, were used as negative controls. DED with HDFs and NHKs were used as a positive control.

At the end of the differentiation protocol, the samples were recovered and washed with PBS. For histological and immunohistochemical evaluation each DED sample was fixed in 10% formalin (at 4˚C overnight), washed in PBS and dehydrated by immersion in 50%, 70%, 90% and finally in 100% ethanol, using an automated tissue processor. After that, the samples were embedded in paraffin, allowed to cool at room temperature and cut at 5μm using a Reichert-Jung 2035 microtome. Sections were picked up on 2% 3-aminopropyltriethoxysilane (APES) in acetone (Sigma).

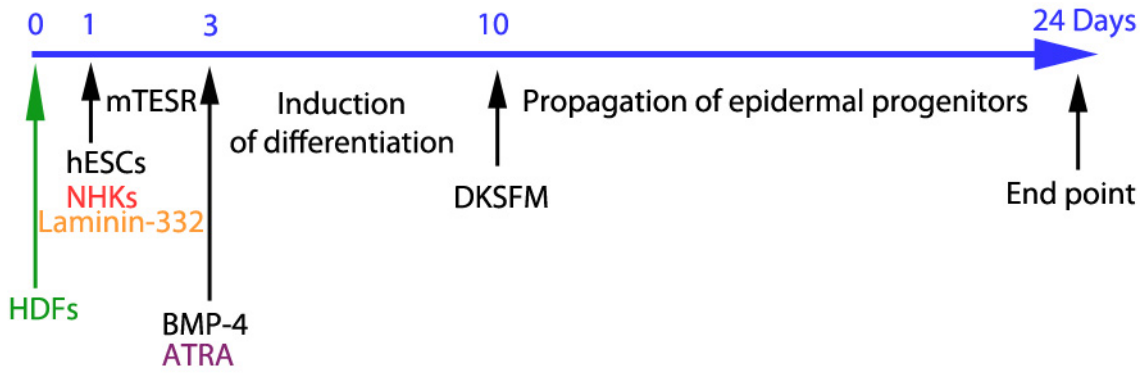

Figure 21 Protocol outline and experimental groups used in DED-induced epidermal differentiation study.
Four experimental groups were tested: 1) DED inoculated with hESCs and differentiation medium containing BMP-4 only; 2) Co-culture of hESCs with NHKs on DED (red); 3) Laminin-332 peptide added to the papillary surface of DED (orange); 4) ATRA added to differentiation medium (purple). Each experimental group was done with or without seeding HDFs on the reticular surface of DED (green).

4.2.6 Haematoxylin & Eosin Y staining of DED sections

After dewaxing (section 2.2.11.1), slides were immersed in a solution of Mayer's haemalum (Sigma) for 3min at room temperature and washed with deionised water. To ensure the appropriate level of nuclear staining, the slides were dipped for 5s in 0.5% HCl in 70% methylated spirit and quickly returned to deionised water, washed for 5min and then examined microscopically. If nuclear staining was too weak, the slides were returned to Mayer's haemalum solution for a further 3min. If nuclear staining was too strong, the slides were dipped again in acid solution. Eosin staining was subsequently performed by immersion in 0.5% eosin Y (VWR) staining solution for 3min at room temperature, sections washed in deionised water. DED sections were mounted by covering with 22x50mm coverslips (VWR), using DPX in Xylene mountant (VWR) as described in section 2.2.11.4. The slides were allowed to dry for 30min and the sections were visualized with

Zeiss Axiophot fluorescence microscope (Zeiss). Images are taken with a CCD camera (DS-U2, Nikon), and processed with Photoshop CS5 (Adobe).

4.2.7 Fluorescence-mediated immunohistochemistry of DED samples

4.2.7.1 De-waxing and antigen retrieval

De-waxing of paraffin embedded sections and subsequent antigen retrieval were performed as described in sections 2.2.11.1 and 2.2.11.2, respectively.

4.2.7.2 Permeabilization

For intracellular antigen staining, permeabilization was performed in 0.6% H_2O_2 (Sigma) and 0.1% Triton X-100 (Sigma) in water for 10min at room temperature. After that, the slides were washed in PBS with gentle agitation (2 times for 5min each). The use of Triton X-100 helps to reduce surface tension, allowing reagents to cover the whole tissue section with ease; it is also believed to dissolve Fc receptors, therefore reducing non-specific binding. The secondary antibody may cross react with endogenous immunoglobulins in the tissue; this was minimized by pre-treating the tissue with a blocking solution containing 0.1% v/v Tween® 20 (Sigma), w/v 1% BSA (Sigma) and 10% v/v normal serum from the species in which the secondary antibody was raised. In this study, all secondary antibodies employed were raised in goat (see section 2.2.7 Table 2 for details), therefore NGS (Invitrogen) was used. The use of normal serum before the application

of the primary also eliminates Fc receptor binding of both the primary and secondary antibody (Daneshtalab et al., 2010). BSA is included to reduce non-specific binding caused by hydrophobic interactions (Boenisch, 2006). Tween 20 is a detergent and surface tension reducer which is frequently used in blocking solutions (Helenius et al., 1979). The slides were then incubated with pre-diluted primary antibodies for 90min at room temperature. The primary antibodies used are shown in Table 9. The slides were then washed in PBS with gentle agitation (2 times for 5min each) and incubated with goat anti-mouse or anti-rabbit Alexa Fluor- conjugated secondary antibodies (1:300 dilution) for 30min at room temperature in the dark. All antibody dilutions were done in blocking solution and a minimum of 80µl of pre-diluted antibodies per section was used. The slides were then washed twice in PBS and a final wash was performed in distilled H_2O for 5min. To mount and counter stain the nuclei, one drop of ProLong® Gold antifade reagent (Invitrogen) with DAPI was added to each section and then glass coverslips (13-mm diameter) were put on top of the slides. The slides were allowed to dry for 30min and the sections were viewed using a with Zeiss Axiophot fluorescence microscope (Zeiss). Images were taken with a CCD camera (DS-U2, Nikon), and processed with Photoshop CS5 (Adobe).

Control procedures, with the omission of either the primary or secondary antibody, were performed to check the specificity of the antigen: antibody interactions.

Antibody	Supplier	Host	Conc.	Protein group
Anti-Keratin 14	Covance	Rabbit	1µg/ml	Basal keratinocyte
Anti-Keratin 5	Covance	Rabbit	1µg/ml	Basal keratinocyte
Anti-Keratin 10	Abcam	Mouse	10µg/ml	Terminal differentiation marker
Anti-Involucrin (SY5)	Sigma	Mouse	1:100	Terminal differentiation marker
Anti-Collagen IV	Sigma	Mouse	1:500	ECM protein
Anti-Collagen VII	Sigma	Mouse	1:1000	ECM protein

Table 9 Primary antibodies used in Chapter 4.

4.2.9 Scoring system for immunohistochemical staining

Relative quantification of immunohistochemical data was done by careful observation of the sections under the fluorescent microscope. The scoring of immunohistochemical staining was done for 4 different factors. Firstly, the staining was evaluated with respect to the appearance of cells in the DED sample. The structures observed could be divided into 4 categories: 1) cellular cysts only, 2) epidermis-like cellular structure inside the DED, 3) a short cellular structure on the surface of the DED, but not directly attached to it, and 4) a long cellular structure attached to the papillary surface of the DED. Representative images for each of the structure types are shown in Figure 22. To estimate the total number of each type of epidermis-like structures observed under each condition, 10 sections of 5µm thickness of each DED sample were evaluated.

In addition, the extent of expression of type VII collagen, type IV collagen and basal keratins 5 and 14 was calculated separately per 5 high-power fields (400X) using a score - to 3+ as follows:

Score	-	+	++	+++
Positive cells (%)	<1%	1-10%	10-50%	50-75%

4.3 RESULTS

4.3.1 hESCs undergo epidermal differentiation in organotypic DED cultures

Histological and immunohistochemical examination of the samples revealed that, when seeded on DED, hESCs filled all pits and holes that are produced in the process of epidermis removal and preparation of DED. In addition, short structures and cysts resembling epithelium were detected. The epithelium-like structures observed could be broadly divided into 4 categories: 1) cystic structures found on the surface and inside the DED (Figure 22, A1 and A2), 2) short cellular structures inside the DED (Figure 22, B1 and B2), 3) short patches of cells detached from the papillary surface of DED (Figure 22, C1 and C2), and 4) long structures attached to the papillary surface of the DED (Figure 22, D1 and D2). "Long" epidermis-like structures were >750μm in length, and comprised around 25% of the papillary DED surface. The majority of cells stained positively for basal keratins 5 and 14 (Figure 23), however no terminal differentiation markers such as keratin 10 and involucrin were detected. Types IV and VII collagen were seen deposited around these presumptive epidermal structures (Figure 23); although, a discrete BM was not observed. It is important to note, however, that neither terminal differentiation markers nor a continuous discrete BM were observed in the positive control sample containing NHKs and HDFs.

Figure 24A and B show the appropriate negative controls for histological and immunohistochemical analysis of the DED samples, respectively.

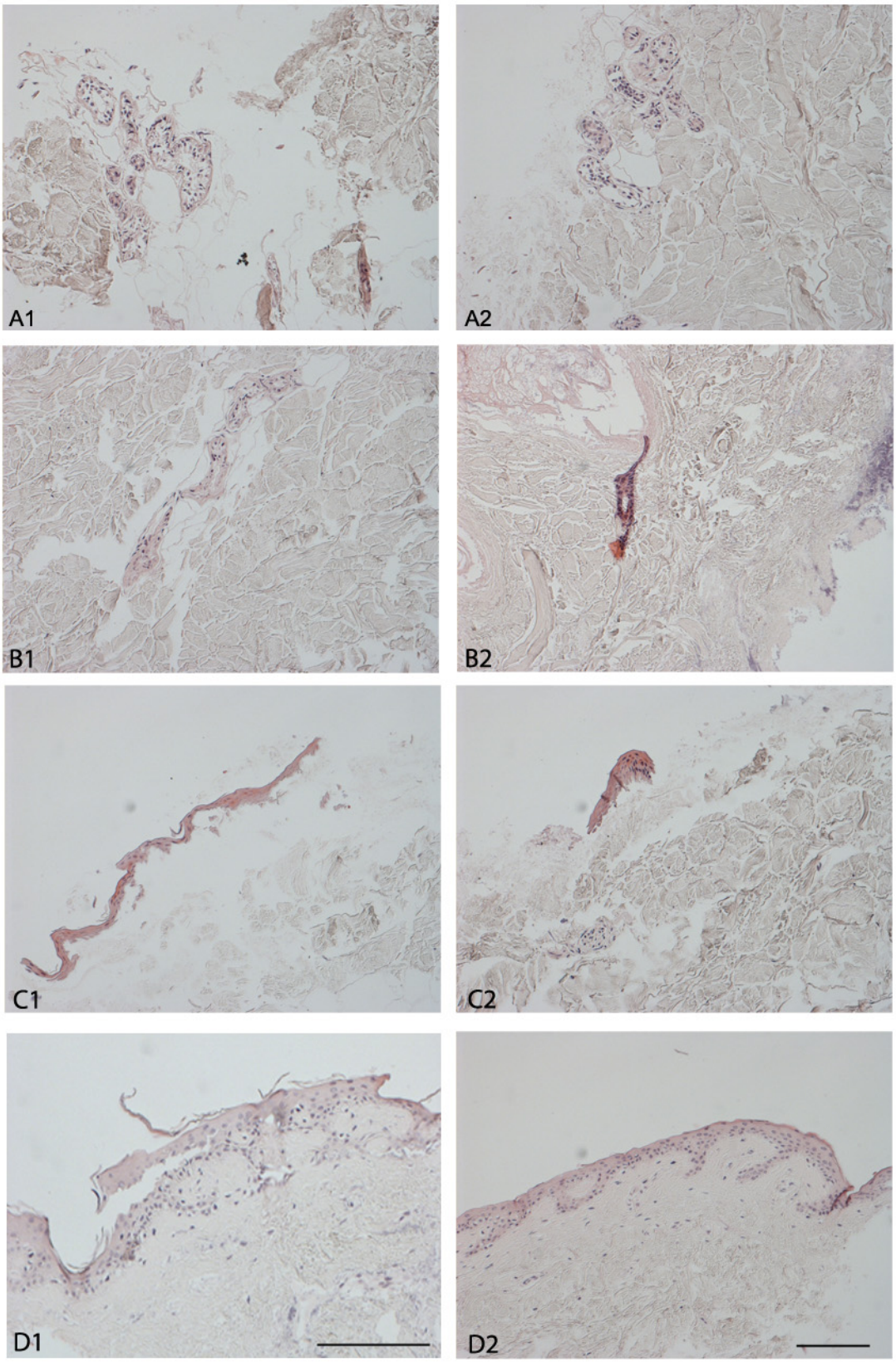

Figure 22 Histological examination of DED samples revealed the presence of 4 different epidermis-like structures formed by hESCs.
A1 and 2: Cystic structures; **B1 and 2:** Epidermal-like structure inside DED; **C1 and 2:** Short patches of cells detached from the papillary surface of DED; **D1 and 2:** A long epidermis-like cellular structure attached to the papillary surface of DED. Scale bar=100μm.

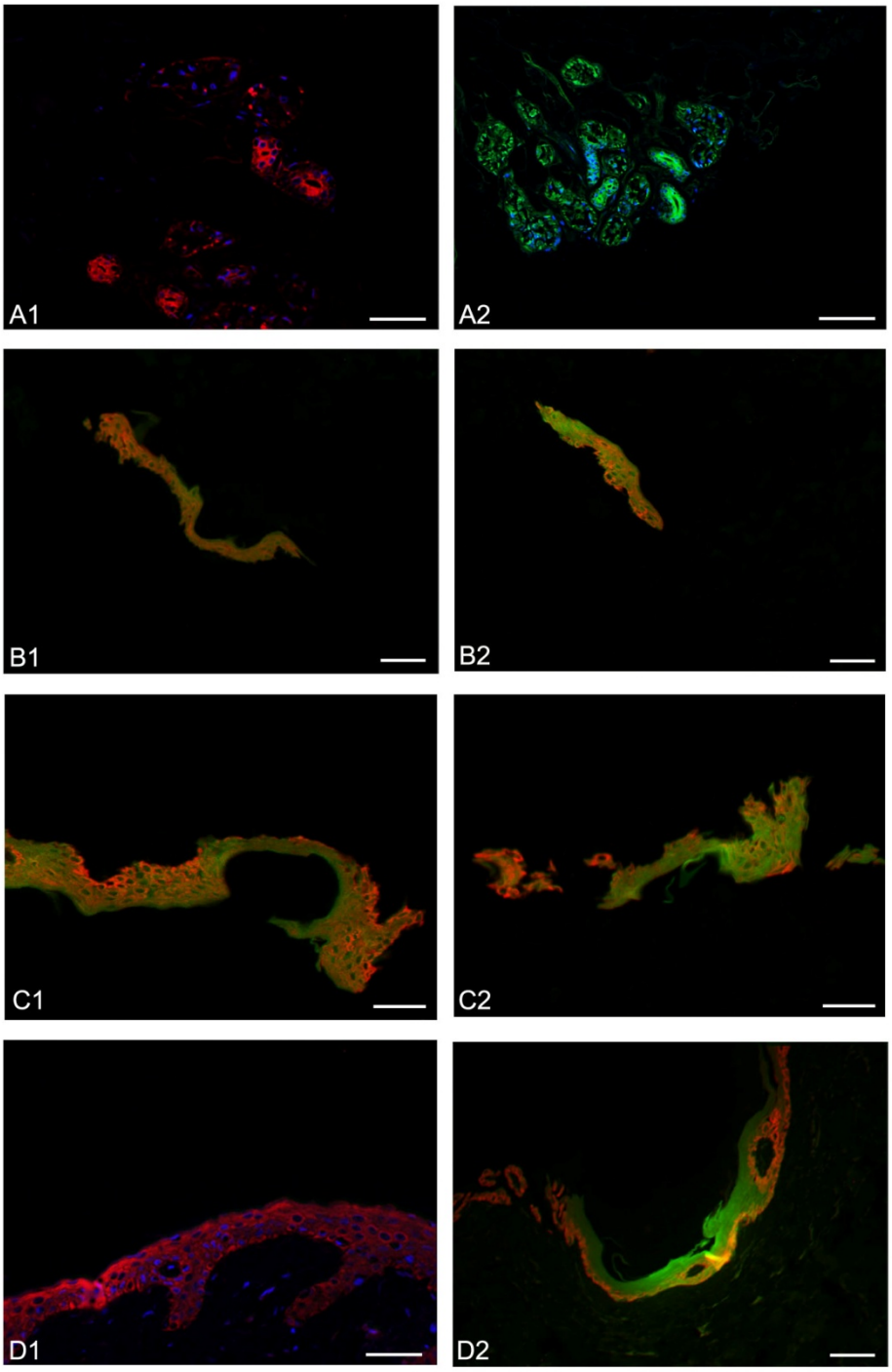

Figure 23 Expression of basal keratins 5 and 14 and ECM proteins in hESC-derived structures in DED samples.
A: A1- The cells within cystic structures stain positive for keratin 5 marker expression (red), and A2- Type IV collagen (green) was seen deposited around the cysts; **B:** Short cellular structures inside DED stain positive for B1- keratin 14 (red) with type IV collagen (green) surrounding the structure, and B2- keratin 5 (red) with type VII collagen (green) deposition; **C:** Epithelium-like patches of cells detached from the papillary surface of DED display positivity for C1- keratin 14 (red) and type IV collagen (green) surrounding the structure, and C2- keratin 5 (red) with type VII collagen (green) deposition; **D:** Long (>700µm) epithelium-like structure attached to the papillary surface of DED stained positive for D1- basal keratin 14 positivity (red) and D2- basal keratin 5 (red) as well as type VII collagen deposition; Scale bars=50µm

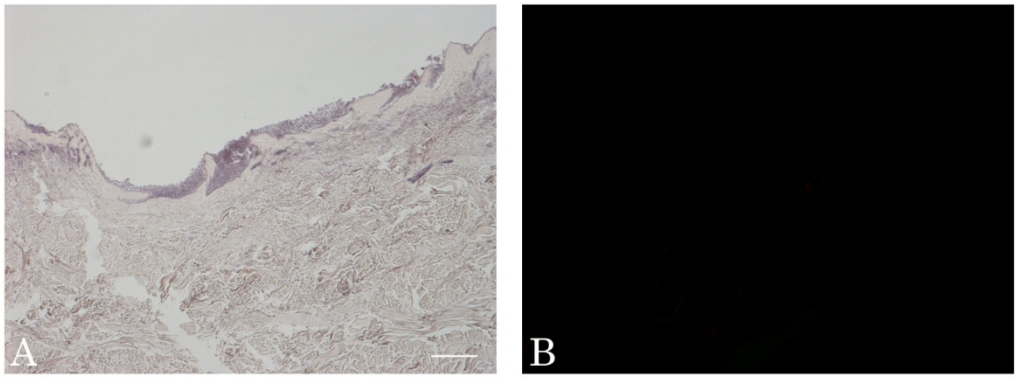

Figure 24 Negative controls for figures 23 and 24.
A: Negative control for histological examination (figure 23) showing DED with HDFs only;
B: Negative control for immunohistochemical analysis (figure 24) showing no primary antibody control. Scale bar=100μm.

4.3.2 Culture conditions involving ATRA and laminin-332 peptide induce epidermal differentiation of hESCs most efficiently

The scoring of immunohistochemistry data is shown in Table 10, 11 and 12. From this analysis, two conditions tested appear to induce epidermal differentiation of hESCs most efficiently, namely addition of ATRA to differentiation medium or application of laminin-332 α3 sequence to the DED surface. Under both of these conditions, long epidermis-like cellular structures (>750μm in length) were observed (Table 10) that were similar to the structures obtained with positive control experiments involving DED populated with NHKs on the papillary surface and HDFs on the reticular surface (Table 12). Furthermore, the expression of types IV and VII collagen and basal epidermal keratins 5 and 14 closely resembled that detected in the positive control sample (Table 11). These epidermis-like structures covered approximately 25% of each section, and were not observed under

any other experimental conditions. When BMP-4 alone was used to induce epidermal differentiation of hESCs, a total of 24 cystic structures were observed in 10x5μm sections examined (Table 10). However, no long epidermis-like structures were detected. Similarly, when undifferentiated hESCs were co-cultured with NHKs in the presence of BMP-4, 18 cystic structures were detected, as well as 6 short structures. Under all experimental conditions tested, the number of more defined epidermal structures as well as expression of epidermal markers increased when the reticular side of DED was inoculated with HDFs.

	Total number of epidermis-like structure per 1mm							
	Cystic	*Inside*	*Short*	*Long*	*Cystic*	*Inside*	*Short*	*Long*
Condition	*-HDFs*				*+HDFs*			
BMP-4 only	24	1	0	0	12	6	0	0
BMP-4+ATRA	18	5	2	0	11	4	9	1
BMP-4+NHKs	18	1	6	0	20	2	1	0
BMP-4+Lam	17	3	0	0	8	0	5	1

Table 10 Quantification of various epidermis-like structures observed under different experimental conditions.
Evaluation of the number of epidermis-like structures reveals that addition of ATRA to differentiation medium or application of laminin-332 α3 sequence to DED surface significantly improves epidermal differentiation from hESCs.

	-HDFs			*+HDFs*		
	ColIV	*ColVII*	*Krt14/5*	*ColIV*	*ColVII*	*Krt14/5*
BMP-4 only	-	+	++	+	+	+
BMP-4+ATRA	++	+	++	+++	+++	+++
BMP-4+NHKs	-	+++	+++	++	++	-
BMP-4+Lam	-	++	++	+++	+++	+++

Table 11 Scoring of immunohistochemical data for epidermal marker expression
Scoring suggests that addition of ATRA or laminin-332 peptide improves epidermal differentiation efficiency of hESCs on DED.

	Epidermis-like structures				*Epidermal markers*		
	Cystic	*Inside*	*Short*	*Long*	*ColIV*	*ColVII*	*Krt14/5*
Positive control	0	0	11	1	+++	+++	+++
Negative control	0	0	0	0	++	+	-

Table 12 The total number of epidermis-like structures and expression of epidermal markers in positive and negative control experiments.

4.3.3 Technical discussion

One potential source of experimental artifacts in the DED experiment described in this chapter is keratinocytes and/or fibroblasts from donor skin which could have survived the process of de-epidermalization and started to proliferate under experimental conditions.

To rule out the possibility of contamination with donor keratinocytes and/or fibroblasts and confirm the hESC origin of the epidermal cells quantitative fluorescent PCR for multiple microsatellite markers of genomic DNA can be applied.

PCR-based techniques allow the origin of the cells to be identified and quantified and have an advantage over cytogenetic Y chromosome probing by FISH as they are not limited exclusively to sex-mismatched transplantations. Microsatellites are highly variable tandemly repeated sequences commonly used for mapping, linkage analysis and to trace inheritance patterns. The repeating unit is generally 1 to 4 nucleotides long and variation in the number of repeats affects the overall length of the microsatellite. Specific primer pairs corresponding to microsatellite markers can be used to generate a profile of PCR amplifiers diagnostic for each genotype. When being used as a diagnostic tool as in preimplantation genetic haplotyping, the specific number of repeats in a given microsatellite is not important, but rather the difference in the number of repeats between alleles, allowing the origin of the allele to be tracked.

Previously, microsatellite analysis of the KCL-002 hESC line and HDFs used in this study has been carried out according to the published quantitative fluorescence PCR protocol (Mann et al., 2004). In the procedure the chromosome markers are co-amplified in one multiplex PCR assay. PCR products are analysed on 310 and 3100 capillary-based genetic analysers (Applied Biosystems, Foster City, CA, USA). Genotyper version 2.5 can be used for sizing and labelling alleles. The results of this analysis are shown

in Table 13. Therefore, to confirm the absence of contamination from donor skin cells, microsatellite analysis of the DED samples can be performed and the results compared to those obtained with two cell lines. Detecting the repeat numbers which correspond only to those in the KCL-002 hESC line and HDFs repeat numbers would confirm the origin of epidermal cells; whereas a new repeat number in the DED samples would indicate contamination by donor cells.

	KCL-002		**HDFs**	
MARKER	*ALLELE 1*	*ALLELE 2*	*ALLELE 1*	*ALLELE 2*
D13S252	299	303	275	295
D13S305	450	450	429	450
D13S325	286	302	281	298
D13S628	457	457	457	
D13S634	401	406	403	411
D18S386	340	359	356	375
D18S390	368	372	360	371
D18S391	214	218	218	228
D18S535	478	482	474	
D18S819	400	412	400	412
D18S978	211	220	215	220
D21S11	241	256	245	
D21S1409	212	229	187	195
D21S1411	305	317	300	313
D21S1435	185	185	189	193
D21S1437	331	335	315	319

Table 13 Microsatellite results for 16 markers from the KCL002 hESC line and HDFs.
The markers are shown grouped for chromosomes 13, 18 and 21.

4.4 DISCUSSION

The results described here suggest that when undifferentiated hESCs are exposed to a complex, tissue-specific microenvironments such as DED of skin, in combination with appropriate differentiation-inducing conditions, the cells can be directed to undergo epidermal differentiation and form epithelium-like structures. However, these structures did not show signs of stratification and did not cover the whole surface of the DED.

4.4.1 Organotypic culture of hESCs in combination with appropriate differentiation-inducing conditions supports the formation of epithelium-like structures

When seeded on DED, hESCs occupied all the pits produced in the process of DED preparation. This observation is in agreement with results obtained when NHKs are cultured on DED (Régnier et al., 1986). No single keratin 14-positive cells were observed and differentiated cells were seen to cluster in groups or patches. Such cell-sorting behaviour has also been described for interspersed populations of dermal fibroblasts and keratinocytes (Wang et al., 2000). This suggests that proximity and inter-communication with neighbouring cells is critical for epidermal differentiation. Four types of epithelium-like structures were detected at the end point of the differentiation protocol that stained positive for basal keratins 5 and 14 expression, although a continuous epidermal layer was not observed. Furthermore, stratification was not detected in experimental groups, as well

as in the positive control sample. Such observations could be explained by the fact that the DEDs studied lacked a complete BM.

Removal of epidermis with PBS has been shown previously to preserve the BM of skin (McKay et al., 1994, Régnier et al., 1984). However, in our study, no discrete BM was observed and ECM proteins accumulated exclusively around hESC-derived structures, suggesting that they were secreted by the differentiating cell population. It has been reported previously that epidermal cells grown on a collagen sponge formed clumps of keratinocytes found in the interior of the sponge instead of a well-organized epidermis (Doillon et al., 1988). Similarly, in another study, a well-organized epidermis was not detected in the C-GAG composites due to a lack of BM (Ojeh et al., 2001). Therefore, it is very likely that the absence of a fully formed BM in DED preparations hinders the successful development of a fully-formed stratified epidermis covering the whole surface of DED. Instead, hESCs invaded the interior of the DED, where they formed epithelium-like structures. The formation of hESC-derived cysts has been previously shown when murine embryoid bodies were seeded on DED (Bagutti et al., 2001). Although in our model embryoid bodies formation was not employed and cells were seeded in a single cell suspension, the tendency of ESCs for spontaneous embryoid body formation when the culture conditions are changed from a two-dimensional monolayer to three-dimensional structure is well known (Keller, 1995, Hopfl et al., 2004, Dang et al., 2002, Itskovitz-Eldor et al., 2000).

4.4.2 Co-culture with NHKs has no effect on epidermal differentiation of hESCs, while co-culture with HDFs enhances the formation of epidermis-like structures

The results obtained in this set of experiments are consistent with previously reported data that co-culture of hESCs with NHKs does not improve the ability of hESCs to undergo epidermal differentiation (Bagutti et al., 2001). Conversely, in all experimental groups, more defined epidermal structures and higher expression of epidermal markers were observed when the reticular side of the DED was inoculated with HDFs. Previous reports also demonstrated that the addition of fibroblasts to organotypic cultures led to the formation of a similar morphology to normal skin (Krejci et al., 1991, Ghosh et al., 1997). This may not be surprising considering the importance of mesenchyme in embryonic epidermogenesis (Sengel, 1976) and the known supportive role of fibroblasts on keratinocyte proliferation (Rheinwald and Green, 1975). Since HDFs remained at the reticular side of the DED and therefore had no direct contact with hESCs, it is highly likely that any differentiation-improving effects were due to the paracrine action of growth factors secreted by fibroblasts. This observation is also in agreement with previous studies on murine ESCs (Bagutti et al., 2001).

4.4.3 ATRA and laminin-332 alpha3 sequence improve the formation of epithelium-like structures from hESCs

Under two experimental conditions tested (namely supplementation of the differentiation medium with ATRA and addition of laminin-332 α3 sequence

to the papillary surface of DED), the formation of epithelium-like structures and marker expression was most efficient, based on scoring of histology and immunohistochemistry data.

Supplementation of the differentiation medium with ATRA significantly improved the formation of epithelium-like structures from hESCs and numerous long cellular structures were observed on the papillary surface of the DED, although only one of them was fully attached to it. Stage-specific application of ATRA has been previously shown to greatly enhance the epidermal differentiation of hESCs (Metallo et al., 2008b), possibly through induction of ΔNp63 expression (Chen and Lohnes, 2005). The known effects of ATRA on keratinocyte proliferation and differentiation are described in more detail in section 1.9.

The addition of laminin-332 peptide to the papillary surface of the DED led to formation of long (>750μm) epithelium-like structures fully attached to the DED. In fact, those structures were equivalent to those obtained in the positive control, when DED was inoculated with NHKs and HDFs. Laminin-332, one of the major constituents of skin BM, forms cross-shaped heterotrimers consisting of α3-, ß3-, and γ2-chains which regulate the stable adhesion of the epidermis to the underlying dermis (Rousselle et al., 1991, Colognato and Yurchenco, 2000) and which influence keratinocyte behaviour through interactions with cell surface integrins α3ß1 and α6ß4 (Rousselle and Aumailley, 1994, Niessen et al., 1994). Furthermore, laminin-332 participates in cellular functions such as adhesion, migration, differentiation, growth, and survival (Nguyen et al., 2000). The laminin α3

chain contains a C-terminal globular (LG) domain that consists of five globular modules LG1–LG5 (Timpl et al., 2000) with the LG3 domain playing an essential role in mediating the unique activity of laminin-332. Earlier studies demonstrated that the motif PPFLMLLKGSTR within the LG3 domain of recombinant human laminin-332 α3 chains is an active site for integrin α3β1 binding (Kim et al., 2005). Previously, the laminin-332 α3 sequence has been shown to promote cellular adhesion (Damodaran et al., 2009) and exert positive effects on neurite growth within a collagen scaffold (Yao et al., 2010). It is, therefore, plausible that the hESCs were encouraged to attach and proliferate on the reticular side of DED and formed epithelium-like structures when induced to differentiate.

4.4.4 Conclusions and future work

Despite producing some epithelium-like structures, hESCs seeded on DED and subjected to treatment with BMP-4 and ATRA did not produce a continuous stratified epidermal layer. This suggests that the differentiation conditions employed failed to completely recapitulate embryonic epidermal morphogenesis. It is very likely that hESCs have to be primed to adopt an ectodermal fate prior to organotypic culture. Previously, murine ESC-derived keratin 18-positive epithelial precursors were co-cultured with fibroblasts at the air-liquid interface and small areas of well-organized reconstituted skin were observed (Coraux et al., 2003a). However, reconstituted skin comprised only 20% of the area, while most of the construct was occupied by zones of uncharacterized non-organized

differentiated cells. The authors suggested that this finding was due to heterogeneity of the starting hESC-derived epithelial cell population. Indeed, the *in vitro* differentiation protocol utilized in this study only yielded 9.8% of keratin 14 positive cells (Coraux et al., 2003a). Therefore, it is plausible that if the efficiency of the *in vitro* differentiation protocol is improved and a keratin 14-positive population of higher purity is obtained, then a complete epidermis could be produced when this population is seeded on DED.

One of the most efficient epidermal differentiation protocols demonstrated thus far was obtained through ATRA treatment of hESCs (Metallo et al., 2008b). These authors reported that after 5 weeks of differentiation 87% of cells expressed keratin 14. These cells were shown to undergo terminal differentiation and stratification when cultured on dermal constructs using rat tail collagen I. However, the profile and strength of terminal differentiation markers differed to that of *in vivo* samples. Furthermore, residual simple epithelial keratin 18 expression, as well as the expression of corneal epithelial markers keratins 3 and 12, was also detected. These observations could reflect the sub-optimal nature of the dermal construct utilized in this study. Studies of human keratinocytes have shown that while elevation to the air-liquid interface *in vitro* stimulates differentiation of the cultivated epidermis, the degree of differentiation is far greater when an appropriate dermal construct is used (Prunieras et al., 1983). DED composites were shown to accurately replicate keratinocyte behaviour in models of wound healing, hyperproliferation, and psoriasis (Leigh et al., 1995, Asselineau et al., 1986). Consequently, DED composites were accepted

to be the organotypic gold standard model (McKay et al., 1994, Prunieras et al., 1983). For that reason, if an efficient production of epidermal progenitors is achieved *in vitro*, it would be favourable to subject these progenitors to an environment that closely mimics human skin, such as DED. Therefore, the work described in the following chapters of this thesis was aimed at developing a more efficient differentiation protocol that could produce a large amount of relatively pure epidermal progenitors *in vitro*.

CHAPTER 5 PA6-STROMAL DERIVED ACTIVITY TO DIFFERENTIATE hESCs TOWARDS EPIDERMAL LINEAGES

HYPOTHESIS:

The sequential contribution of mesenchymal inducers such as stromal feeders and type IV collagen can replicate *in vitro* the effects of embryonic mesodermal stimulation and encourage epidermal differentiation from hESCs. To test this hypothesis, we used the medium enriched in growth factors secreted by the PA6 stromal cell line in combination with type IV collagen as substrate since this combination might display mesenchymal inducing activity *in vivo*.

AIMS:

1. Adapt and optimize and evaluate the efficiency of the existing direct co-culture differentiation protocol for the KCL-002 hESC line.
2. Evaluate the efficiency of epidermal differentiation promoted by growth factors secreted by the PA6 stromal line by monitoring of epidermal marker expression.
3. Examine whether PA6 growth factors can exert similar effects on epidermal differentiation of hESCs to the direct co-culture method.

5.1 INTRODUCTION

The rationale behind the hypothesis outlined in this chapter relies on the assumption that using mesenchymal inducers such as stromal feeders and type IV collagen to direct hESCs differentiation *in vitro* has the potential to mimic the effects of embryonic mesodermal stimulation and encourage epidermogenesis.

5.1.1 PA6 cells and Stromal-Derived Inducing Activity (SDIA)

The instructive effect of mesenchymal factors on epidermal commitment correlates well with the induction role of mesodermal cells on ectodermal fate during embryonic skin development and post-natally (Sengel, 1976). Stromal cell lines such as PA6, derived from mouse bone marrow, have been shown to exhibit what has been termed stromal-derived inducing activity (SDIA) (Kawasaki et al., 2000). It has been well documented that this activity can induce ectodermal differentiation of hESCs, which in the absence of other stimuli proceeds towards neural cell fate, and can serve as an efficient protocol for obtaining mesencephalic dopaminergic neurons (required for cell therapy of Parkinsonism) (Buytaert-Hoefen et al., 2004, Kawasaki et al., 2000, Kawasaki et al., 2002, Yan et al., 2005, Zeng et al., 2004a). Despite numerous studies, the nature of SDIA, however, remains unclear. Initial observations indicated that PFA-fixed PA6 cells can induce neural differentiation from hESCs, thus suggesting that SDIA accumulates on the surface of PA6 cells (Kawasaki et al., 2000). For example, PA6 cells express the Notch ligand Jag1 and Notch signalling has been reported to

direct ESCs differentiation towards neuroectoderm (Lowell et al., 2006). Therefore, it is likely that Jagged1 is a key component of SDIA, and that Notch activation in ESCs by PA6-expressed Jag1 favours their differentiation towards the neuroectodermal lineage.

On the other hand, several studies have shown that soluble factors secreted by PA6 cells also play a role in inducing neural differentiation from hESCs (Schwartz et al., 2005, Yamazoe et al., 2005). Genome expression analysis study has attempted to identify factors secreted by PA6 cells that might be effective in inducing neural differentiation from hESCs (Vazin et al., 2009). In this study, four factors were found to be responsible for PA6 cell-derived inducing activity: Stromal cell-derived factor 1, Pleiotrophin, Insulin-like growth factor 2, and Ephrin B1. The authors concluded that the combination of these four factors, termed SPIE, was primarily involved in promoting SDIA, whereas the cell surface activity of PA6 cells enhanced cell survival and overall neurogenesis.

However, a different study carried out by the same group has shown that medium conditioned by PA6 stromal cells (PA6 conditioned medium; PA6 CM) was not as efficient at inducing dopaminergic neurons differentiation from hESCs compared to live PA6 cells, although it did promote their survival with sustained Oct-3/4 expression (Vazin et al., 2008). Interestingly, the same study revealed that SDIA promoted by PA6 cells is drastically decreased by both fixation and irradiation but is less affected by mitomycin C treatment; conversely, the neural-inducing effect is not altered by fixation but is decreased by mitotic inactivation. These collective results

suggest that it is very likely that SDIA is in fact mediated by the combination of cell surface activity and soluble factors secreted by PA6 cells.

5.1.2 BMP-4 as an antagonist of neural fate

It has been shown that SDIA-treated ESCs tend to adopt the default neural fate unless they receive a considerable level of BMP signalling (Kawasaki et al., 2000). In their original study Kawasaki *et al.* (2000) showed that >90% SDIA-treated ESCs differentiate efficiently into neural precursors and neurons unless exogenous BMP-4 is added.

BMPs are members of the transforming growth factor-ß superfamily. In *Xenopus,* BMP-4 plays a pivotal role in establishing the dorsal-ventral axis of early embryos: in the ventral part of the egg BMP-4 induces the epidermal commitment, while its absence within the dorsal part leads to a "default neural" differentiation (Wilson and Hemmati-Brivanlou, 1995). In vertebrate embryogenesis, the primordial nervous system arises from uncommitted ectoderm during gastrulation. Spemann and Mangold (1942) demonstrated that the dorsal lip of the amphibian blastopore, which gives rise mainly to axial mesoderm, emanates inductive factors that direct neural differentiation in ectoderm. Molecular studies in *Xenopus* have identified neural inducer molecules and revealed their mode of action (Sasai and De Robertis, 1997). Neural inducers such as noggin and chordin (Lamb et al., 1993, Sasai et al., 1995) induce neural differentiation in isolated *Xenopus* ectoderm (animal caps) and promote dorsalization of mesoderm when acting on mesodermal precursors (Sasai and De Robertis, 1997). These

neural inducers do not have their own receptors on target cells, instead they act by binding to and inactivating BMP, which suppresses neural differentiation and ventralizes mesoderm (Wilson and Hemmati-Brivanlou, 1995, Piccolo et al., 1996). These results suggest that both neural induction and mesoderm dorzalization are controlled by a common morphogenic signalling, that is, a BMP activity gradient (Sasai, 2000). Similarly, in vertebrates, including humans, neural/epidermal binary decision is executed in a BMP-dependent manner, where BMP-4 has a critical role in epidermal commitment and displays an inhibitory effect on neural induction (Hemmati-Brivanlou and Melton, 1997).

These effects of BMP-4 are achieved through the Smad-mediated apoptosis of neural precursors (Gambaro et al., 2006). The original paper on SDIA promoted by PA6 co-culture showed that BMP-4 treatment of ESCs significantly increased expression of the non-neural ectoderm marker, E-cadherin, from 16% to 75% (Kawasaki et al., 2000). Furthermore, after 11 days of culture the expression of keratin 14 increased from 0% to 34%. The authors also demonstrated that addition of serum during days 3-5 of the induction period further promoted epidermogenesis and generated large colonies with a strong keratin 14 signal (47%). Importantly, mesodermal induction did not occur in these conditions.

Therefore, the possibility arises that combining optimal PA6 stromal cells co-culture and BMP-4 treatment might efficiently direct hESCs towards epidermal fate and produce keratinocyte precursors.

5.1.3 Keratins as markers to monitor the efficiency of epidermal differentiation of hESCs *in vitro*

In 2008, Aberdam *et al.* developed a method to produce a homogenous ectodermal precursor cell population (Aberdam et al., 2008). Their approach involved seeding undifferentiated hESCs on formaldehyde-fixed PA6 stromal feeder cells in the absence of serum for 4 days and exposed to 0.5nM BMP-4 treatment for 3 days, followed by an additional 7 days of culturing in the presence of 10% foetal calf serum. Under these conditions, a large number of keratin 18-positive ectodermal progenitor cells were produced (60%), along with keratin 5/keratin 14-positive keratinocytes (20%). A related protocol involved subculturing the ectodermal progenitors obtained at day 7 on mouse embryonic fibroblast feeders in medium supplemented with 5µg/ml insulin, 0.5µg/ml hydrocortisone, 50µg/ml ascorbate, and 10ng/ml recombinant human epidermal growth factor for another week. This procedure yielded a homogeneous population of ectodermal cells, 100% positive for keratin 18. These cells could be further expanded on type IV collagen and cultured for 15 passages, while maintaining homogeneity for keratin 18 staining. This work generated for the first time a stable, somatic ectodermal cell population 100% positive for keratin 18. The authors proposed that such ectodermal cell population could be particularly useful for designing *in vitro* models to recapitulate early embryonic epidermogenesis and lineage specification, as well as a potential source for further derivation of epidermal stem cells for clinical trials (Aberdam et al., 2008).

Despite its homogeneity, the cell population obtained was 100% positive for the expression of keratin 18, a marker of simple single-layer epithelia (Romano et al., 1986). Keratin 18 and its pairing partner keratin 8 are the earliest keratins to be expressed in the preimplantation embryo (Jackson et al., 1980, Oshima et al., 1986). As the epidermis begins to differentiate, expression of simple epithelial keratins is lost from presumptive keratinocytes and restricted to periderm (Dale et al., 1985) and then lost altogether from skin as the periderm is lost following stratification. Post-natally, keratins 8/18 expression remains in a variety of tissues where simple epithelial lining is present, such as colon, kidney, pancreas, salivary glands, liver and placenta (Moll et al., 1982). Aberrant keratin 8/18 expression in adult skin can be seen in malignancies such as squamous cell carcinoma (Markey et al., 1991, Wu and Rheinwald, 1981)

On the contrary, the authors reported only 20% positivity for the expression of basal keratin 14 (Aberdam et al., 2008). Further maturation to keratin 14-positive cells from the keratin 18-positive ectodermal precursors, however, was achieved by transduction with a lentivirus expressing ΔNp63 (Aberdam et al., 2008). Spontaneous keratin 14 expression was also observed when the ectodermal precursor population was seeded onto a HaCaT (human keratinocyte cell line)-derived ECM (Aberdam et al., 2008).

Therefore, the first objective of this set of my experiments was to adapt and replicate the direct co-culture with PA6 cells for hESC line KCL-002 and promote further differentiation to keratin 14-positive epidermal precursors without the use of viral constructs. Optimization of the protocol was

required because both the spontaneous and lineage-specific differentiation potential varies among different hESC lines due to a variety of factors such as the derivation method, embryo quality and culture conditions (Allegrucci and Young, 2007, Skottman et al., 2005). For this purpose, different direct co-culture methods were evaluated by morphological criteria. In order to promote epidermal differentiation towards keratin 14-positive cells, ectodermal progenitors obtained at day 14 were selected for their rapid adhesion to type IV collagen (Dong et al., 2007) and subcultured in DKSFM (Invitrogen) for a further week. It was then hypothesized that PA6 CM in combination with BMP-4 can efficiently direct hESCs towards epidermal cell lineages. This hypothesis relies on the assumption that growth factors secreted by PA6 CM could promote the same level of SDIA as intact PA6 cells and, in combination with BMP-4 and type IV collagen, could sufficiently induce epidermal differentiation of hESCs. To test this hypothesis the effects of a) pooled PA6 CM and b) indirect co-culture method using permeable support Transwell® Systems (Corning) were examined.

5.1.4 Indirect co-culture studies

Haematopoietic (Tian et al., 2004), osteogenic (Tong et al., 2007) and chondrogenic (Vats et al., 2006) differentiation of hESCs and pancreatic differentiation in mouse ESCs (Uroic et al., 2010) have been successfully achieved with permeable support Transwell Systems. These permeable supports allow cells to be grown in a polarized state *in vitro* and permit the uptake and secretion of molecules on both basal and apical cell surfaces,

thereby replicating metabolic activities in a more natural fashion. In indirect co-culture the two cell types are grown in different compartments of a Transwell System separated by a microporous 0.4μm membrane (Figure 25). Polyester membrane is microscopically transparent, thus allowing assessment of cell viability under a phase-contrast microscope. The advantage of an indirect co-culture method over PA6 CM alone is that the permeable support of a Transwell insert can continuously provide undifferentiated hESCs with higher amounts of newly-synthesized factors. In addition, even though the pore size would not allow any direct contact between the two cell types, some contribution of adhesion molecules, integrins, and other cell-surface proteins on the stromal PA6 cells may still stimulate important activation pathways.

The overall goal of this set of experiments was to evaluate and compare the efficiency of epidermal differentiation of hESCs induced by SCID exerted by PA6 cells using two different methods, the first one relying on PA6 CM alone (Figure 26B) and the second utilizing an indirect co-culture method using a permeable Transwell System (Figure 26C).

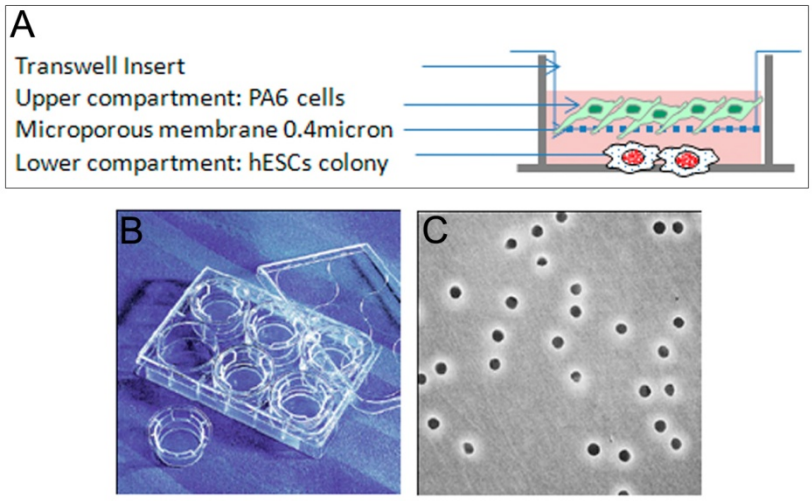

Figure 25 Indirect co-culture of hESCs and PA6 stromal cells in the Transwell System.
A: Schematic representation of indirect co-culture using the Transwell System. hESCs are seeded in the lower compartment at the bottom of the well. PA6 cells are seeded onto the Transwell insert in the upper compartment. Microporous 0.4µm membrane separates the two compartments; **B:** Six-well plate with Transwell inserts; **C:** Electron photomicrograph showing the structure of a 0.4µm polyester pore. B and C are taken from Corning Inc Life Science, "Transwell Permeable support" guide

5.2 METHODS

5.2.1 PA6 cell culture: maintenance and propagation

PA6 cells (Riken Cell Bank, Ibaraki, Japan) were grown in cell culture flasks (Appleton Woods) at a density of $2x10^6$ cells per $75cm^2$ flask and cultured in DMEM (Invitrogen) supplemented with 10% FBS (PA6 culture medium). Approximately every 5 days, subconfluent (cca 80% confluency) cultures were passaged as follows. The medium was aspirated and the flasks were rinsed with Ca^{2+} and Mg^{2+}-free PBS (Invitrogen). TrypLE™ Express (Invitrogen) was added to the flasks and incubated for 5min at 37°C. After that, PA6 culture medium was added and the cell aggregates were broken down by gentle pipetting. The cell suspension was then

transfered into a 15ml conical tube and centrifuged at 400g for 5min. The medium was aspirated and the pellet resuspended in PA6 culture medium according to the desired split ratio. Cells were grown in a 37°C incubator at 5% CO_2 until confluent.

5.2.2 Preparation of PA6 CM

For PA6 CM preparation, subconfluent (cca 80%) cultures of PA6 cells were used. These cultures were obtained by plating $2x10^6$ cells per 75cm² flask and growing them for 5 days in PA6 culture medium. CM was prepared by incubating subconfluent PA6 cells in 85% DMEM:Ham's F-12 medium (1:1) (Invitrogen) and 15% Ko-SR (Invitrogen) for 24 hours. Pooled PA6 CM were filtered to remove cell debris and stored at -20°C for a maximum of 2 days before use.

5.2.3 Preparation of PA6 feeder layers

PA6 cells were seeded at $3x10^5$ cells per well in collagen type I-coated 24-well plates and allowed to attach overnight.

Fixation with 4% PFA was done as follows. Plates were rinsed with PBS and 500µl of 4% PFA per well was added for 10min at room temperature. Plates were then rinsed twice with PBS before seeding hESCs. Mitomycin C treatment: 500µl of mitomycin C (Sigma) diluted in DMEM:Ham F-12 (1:1) (Invitrogen) to a concentration of 10µg/ml was used per well. The plates were incubated for 3 hours at 37°C and rinsed thoroughly with PBS (three

times) before seeding hESCs. In the case of live co-culture, hESCs were seeded directly onto PA6 feeders. Undifferentiated hESC colonies were passaged enzymatically, as described previously (section 2.2.5) and seeded at a density of $10x10^3$ cells per cm^2 for all experiments.

5.2.4 Direct co-culture differentiation

The timeline of the direct co-culture differentiation is outlined in Figure 26A. Undifferentiated hESC colonies were passaged enzymatically using dispase and seeded at a density of $15x10^3$ cells per well in 24-well plates. The colonies were cultured in 84% DMEM:Ham's F-12 medium (1:1) (Invitrogen), 15% Ko-SR (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1mM ß-mercaptoethanol (Invitrogen) for 3 days. At day 4, 0.5nM human recombinant BMP-4 (R&D Systems) was added to the culture medium for a further 3 days. At day 7, BMP-4 was removed, and cells were cultured for one week in differentiation medium composed of 60% DMEM, 30% Ham's F-12 medium, and 10% FBS, supplemented with 5µg/ml insulin, 0.5µg/ml hydrocortisone, 50µg/ml ascorbate, and 10ng/ml recombinant human epidermal growth factor. The putative ectodermal precursors obtained at this stage were selected for their rapid adherence to type IV collagen (described below) and further expanded in DKSFM (Invitrogen).

The differentiation protocol was independently repeated 3 times with hESCs at different passage number (n=3 biological replicates).

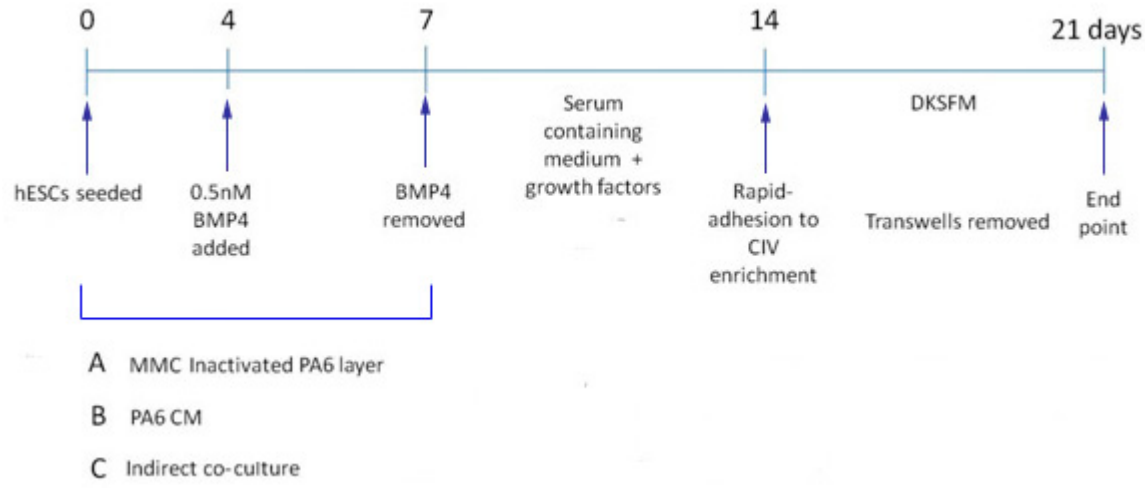

Figure 26 Schematic representation of differentiation protocols described in this chapter, relying on SDIA of the PA6 cell line.
A: Direct co-culture method; **B:** PA6 CM method; **C:** Indirect co-culture method
MMC- mitomycin C; CM- PA6 conditioned medium; CIV- type IV collagen; DKSFM- Defined Keratinocyte Serum Free Medium

5.2.5 Rapid Adhesion Enrichment method

Type IV collagen from human placenta (BD) was reconstituted to a stock concentration of 1.0mg/ml in 10mM acetic acid. The reconstituted solution was allowed to dissolve for 3-5 hours at 4°C, swirling occasionally. For coating, type IV collagen solution was added to culture dishes to a final concentration of 5µg/cm^2. The plates were incubated for 1 hour at room temperature and washed thoroughly with distilled H_2O to remove any remaining acetic acid before plating the cells. The cells were allowed to attach for 15min at 37°C before being washed until there were no moving cells seen under the phase contrast microscope. After fresh DKSFM was added to the culture plates, the remaining cells were returned to the incubator and allowed to grow until further analysis.

5.2.6 PA6 CM-induced differentiation

The protocol of differentiation using PA6 CM is shown in Figure 26B. Undifferentiated hESCs were passaged enzymatically and seeded onto type IV collagen-coated plates at the density described above. The cells were grown in PA6 CM alone for 3 days with daily media changes because of possible degradation of secreted factors. At day 4, 0.5nM human recombinant BMP-4 was added to the culture medium. At day 7, BMP-4 was removed, and cells were cultured for one week in the differentiation medium, as described in direct co-culture method. At the end of this stage, ectodermal progenitors were expanded as described in the "Rapid adhesion enrichment" method section 4.2.5 and allowed to grow for a further week before being fixed in methanol or 4% PFA for immunofluorescence analysis.

The differentiation protocol was independently repeated 3 times with hESCs at different passage number (n=3 biological replicates).

5.2.7 Indirect co-culture differentiation using Transwell® Systems

The indirect co-culture protocol outline is shown in Figure 26C. PA6 cells were collected by trypsinization and seeded onto polyester 0.4µm Transwell insets (Corning, supplied by Appleton Woods) in 6-well plates with insert membrane growth area of 4.67cm^2 (Figure 25). PA6 cells were seeded at a density of 2,000/cm^2 in 1.5ml of PA6 culture medium and allowed to adhere overnight. Two days prior to adding the PA6-containing Transwell inserts,

hESCs were enzymatically passaged onto type IV collagen-coated 6-well plates and cultured in hESC medium. After the addition of the inserts, the medium was changed to DMEM:Ham's F-12 (1:1) (Invitrogen), 15% KSR (Invitrogen) medium. After 3 days, 0.5nM BMP-4 was added and media changes were performed daily for a further 3 days. At day 7, Transwell inserts were discarded and differentiating cells were cultured for a further week in differentiation medium, as described previously. At that point, the putative epithelial sheets were subcultured on type IV collagen (as described in "Rapid adhesion enrichment" method section 4.2.5) and maintained for a further week prior to immunocytochemical analysis and flow cytometry and RNA extraction.

The differentiation protocol was independently repeated 3 times with hESCs at different passage number (n=3 biological replicates).

5.2.8 Immunocytochemistry

In order to examine the expression of epidermal-associated proteins, cells at different time points of differentiation were fixed and processed for immunocytochemistry. At each time point 3 wells of a 24 well plate were randomly chosen for immunocytochemical analysis (3 technical replicates). Fixation was performed with ice-cold methanol at 4°C for 10min, which allows for simultaneous permeabilization of the cell membrane. For α6-integrin staining, permeabilization of the cell membrane was not employed and the cells were fixed with 4% PFA for 10min at room temperature. The protocol was then as described in section 2.2.7. Primary antibodies used in

this chapter are detailed in Table 14. Secondary antibodies are described in section 2.2.7. Figure 34 shows representative images for no primary antibody negative controls for antibodies used in this chapter.

Antibody	Supplier	Host	Species reactivity	Isotype	Concentration	Antigen Localization
Anti-Keratin 14	Covance	Rabbit	Human, Mouse	IgG	1µg/ml	Intracellular
Anti-Keratin 5	Covance	Rabbit	Human, Mouse	IgG	1µg/ml	Intracellular
Anti-Keratin 18	Chemicon	Mouse	Human	IgG1	20µg/ml	Intracellular
Anti-Integrin alpha6	Abcam	Mouse	Human	IgG2b	1µg/ml	Transmembrane
Anti-Sox2 (Y-17)	Santa Cruz	Goat	Human, Mouse, Rat	IgG	1µg/ml	Intranuclear

Table 14 Primary antibodies used in Chapter 5.

5.2.9 Quantification of immunofluorescence data

Quantification of immunofluorescence data was carried out by counting cells showing a positive fluorescence signal for keratin 14 in 3 independent fields chosen at random containing 100 cells at two time points, day 14 and 21. Data are presented as percentage of cell numbers in relation to the total number of DAPI-positive cells. Quantification was carried out in 3 independent experiments. Values are presented as mean ± Standard Error of the Mean (SEM). An inferential error bar was used in this analysis in order to more accurately interpret the data. SEM= SD/√n and represents a measure of how variable the mean will be, if the study is repeated many times. An unpaired two-tailed Student's t-test was used to analyze data for statistical significance for keratin 14 protein expression between two differentiation protocols at two time points. Graphs and analysis were

generated with GraphPad Prism (Version 5) Software. A p value < 0.05 was considered to be statistically significant.

5.2.10 Indirect Flow cytometry for keratin 14 staining

The cells were harvested by trypsinization using TrypLE Express (Invitrogen) and centrifugation at 300g for 5min. The cell suspension was adjusted to a concentration of 0.5-1x10^6 cells/ml in ice-cold *Flow Cytometry* (FC) buffer (1% BSA/PBS) and 100µl of cell suspension was added to each sample tube. Polystyrene round-bottom 12x75 mm Falcon tubes (BD) were used for cell staining. A minimum of 10^5 cells/per sample was analyzed. To fix the cells 100µl of 4% (v/v) PFA in PBS was added to each sample tube and kept for 10min at 4°C. The cells were then washed with 2ml FC buffer at 500g for 5min. The supernatant was then poured off leaving approximately 100µl volume containing the cell pellet. For intracellular keratin staining, each sample was permeabilized with 500µl ice-cold 0.1% Triton-X 100/PBS and kept on ice for 20 min. The samples were then washed with 2ml FC buffer at 500g for 5min. The cells become more buoyant after permeabilization and much care had be exercised to maintain the volume of cells. The supernatant was poured off and 1µg of pre-diluted primary rabbit anti-keratin 14 antibody (Covance, PRB-155P) in 20µl FC buffer was added to each sample. The antibody dilutions were made using 3% BSA/PBS. The tubes were vortexed and incubated for 45min at room temperature. The samples were then washed with 2ml FC buffer at 500g for 5min. The supernatant was poured off and 1µg of pre-diluted Alexa-Fluor 488 labelled secondary antibody (Invitrogen) in 100µl FC buffer was added

to each sample. The tubes were vortexed and incubated for 45min at room temperature in the dark and then washed with 2ml FC buffer at 500g for 5min. The supernatant was poured off and the cell pellet was resuspended in 500µl acquisition buffer composed of 1% BSA and 0.1% (w/v) sodium azide in PBS. The cells were kept on ice until analysis. The data were acquired using a BD FACSCanto™ II Flow Cytometer (BD, Oxford) within 12 hours.

An unstained control with no primary or secondary antibodies was used as a negative control to determine background autofluorescence. Unstained cells were resuspended and washed, as described for stained samples. This control was used to set the voltage level for the scatter and fluorescence channels.

Undiffrentiated hESCs (day 0 of differentiation) served as a negative control. NHKs were used as a positive control to set up cytometer alignment and to remove spectral overlap.

5.2.11 Quantitative PCR

A detailed protocol is described in section 2.2.8. RNA samples were collected at day 7, 14 and 30 of differentiation from 3 independently performed experiments (n=3 biological replicates). In addition, for all genes of interest, quantitative PCR was performed with cDNA from undifferentiated KCL-002 hESC colonies. Copy numbers of all genes of interest were expressed as a ratio against GAPDH copy numbers and then extrapolated against copy

numbers in undifferentiated hESCs, which were taken to equal 1. The Primer3 website (http://frodo.wi.mit.edu/) was used to design the primers. The optimal annealing and extension temperature of the DNA Polymerase contained in the SYBR® Green PCR Master Mix (Roche) is 60°C, hence primers had to be chosen that were compatible with reagents to be used. Primers were designed with a maximum product size of 250 base pairs (bp). Specificity of the primer pairs for relevant genes was verified by performing nucleotide alignment searches using BLAST. All primers pairs were obtained from Sigma. The primers and positive cDNA samples for standard curve derivations are shown in Table 15.

5.2.12 Population Doubling assay

Putative keratinocyte precursors (pKPCs) obtained at day 25 were subcultured on type IV collagen-coated 6-well plates (BD Biosciences) in DKSFM at a density of 5,000 cells per well. The cells in the duplicate plates were harvested and counted every 24 hours for 25 days and the results were plotted on a log-linear scale.

The population-doubling time was determined by identifying a cell number along the exponential phase of the curve, tracing the curve until that number had doubled, and then calculating the time between the two.

Gene	Acronym	Primer sequence	Tm (°C)	Positive control cDNA
Keratin 14	*KRT14*	Forward: 5'GGCCTGCTGAGATCAAAGAC-3' Reverse: 5'-TGTCTCATACTTGGTGCGGA-3'	63.9 64.4	Whole human skin
Keratin 18	*KRT18*	Forward: 5'-AAGGCCTACAAGCCCAGATT -3' Reverse: 5'-CAGTGTGGTGCTCTCCTCAA-3'	63.7 64.3	Whole human skin
Involucrin	*hINV*	Forward: 5'-GATGTCCCAGCAACACACAC-3' Reverse: 5'-TGCTCACATTCTTGCTCAGG-3'	64.3 64.2	Whole human skin
Glyceraldehyde- 3-phosphate dehydrogenase	*GAPDH*	Forward: 5'-GAAGGTGAAGGTCGGAGTC-3' Reverse: 5'-GAAGATGGTGATGGGATTTC-3'	60.0 60.0	Human foetal brain cell

Table 15 Primers used in quantitative PCR reactions in co-culture set of experiments. Tm = melting temperature. * These cDNA samples were used to derive standard curves for the expression of the relevant gene.

5.3 RESULTS

5.3.1 Direct PA6 co-culture supports KCL-002 differentiation into early epidermal precursors

5.3.1.1 Comparison of direct co-culture methods using morphological criteria

The rationale behind comparing different co-culture methods was to establish which approach most efficiently preserves the viability of hESC colonies, while still supporting a satisfactory level of SDIA promoted by PA6 stromal cells. The results are presented in Figure 27. The morphology of hESC colonies suggested that the fixation method was too aggressive for the KCL-002 cell line and that the residual PFA had toxic effects on hESCs, resulting in increased cell death. Live co-culture also had detrimental effects on hESCs proliferation. In general, live co-culture is sub-optimal due to the increased difficulty of separating of potential epidermal precursors from PA6 feeders. Mitomycin C-treated PA6 feeder layers appeared to be the most effective in maintaining survival and proliferation of hESCs. Therefore, for my subsequent experiment mitomycin C-treatment of PA6 cells was chosen as the preferred co-culture method.

Having chosen the method for direct co-culture differentiation approach, the next step was to evaluate the efficiency of differentiation by immunocytochemistry.

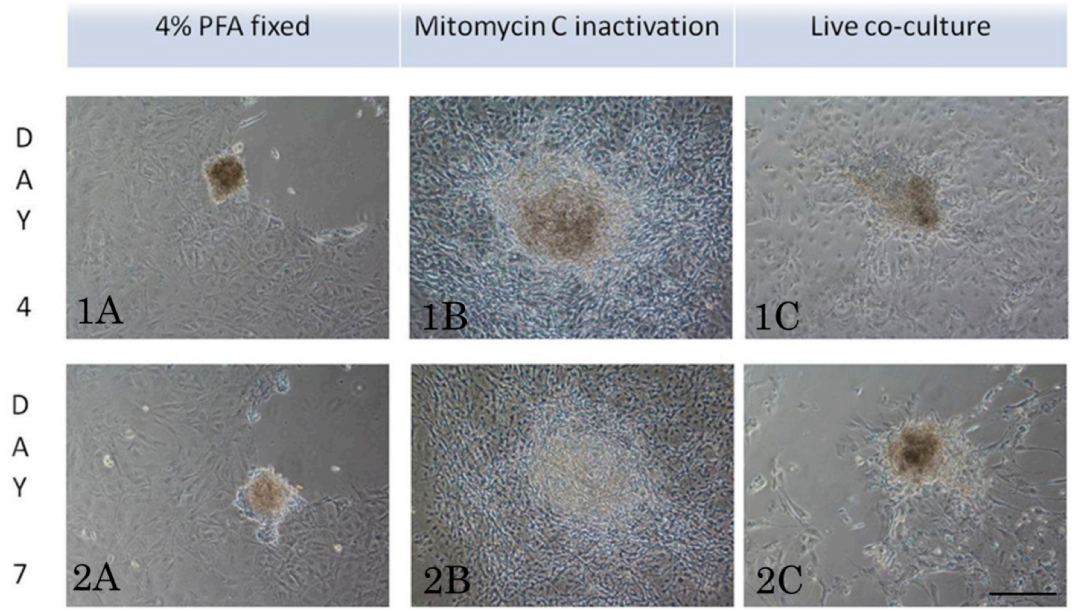

Figure 27 Comparison of different methods for direct co-culture of the KCL-002 hESC line with the PA6 stromal cell line based on morphological criteria at 2 time points: day 4 (time point 1) and day 7 (time point 2).
The top horizontal panel shows the three methods tested: fixation with 4% PFA (A), inactivation with mitomycin C (B) and live co-culture (C). **1A and 2A:** In 4% PFA treated PA6 feeder-layer apparent toxic effects on KCL-002 hESC colonies resulted in icreased cell death; **1B and 2B:** Mitomycin C inactivation of PA6 cells appears to encourage hESC colony growth and expansion; **1C and 2C:** Live co-culture with PA6 feeders is detrimental to hESCs with decreased cell surivival and proliferation. Scale bar=100µm.

5.3.1.2 Expression of epidermal markers

In order to assess the ability of the KCL-002 hESC line to undergo epidermal differentiation during direct co-culture with the PA6 stromal cell line, differentiating colonies were tested for the presence of keratins by immunocytochemistry. Immunofluorescence analysis at day 14 showed that the colonies were positive for simple epithelial keratin 18, while some basal keratin 5 expression was also observed (Figure 28). This suggests that the early ectodermal precursors have started developing into a more defined epidermal population. Following enrichment and subculture, the expanded progenitors formed homogenous populations of pKPCs positive for keratin 14 at day 21 (Figure 29). These results confirm that the KCL-002 hESC line

could undergo epidermal differentiation in response to SDIA of PA6 cells. The next aim was to see if growth factors within the medium conditioned by PA6 cells could exhibit a similar level of SDIA on KCL-002 hESCs.

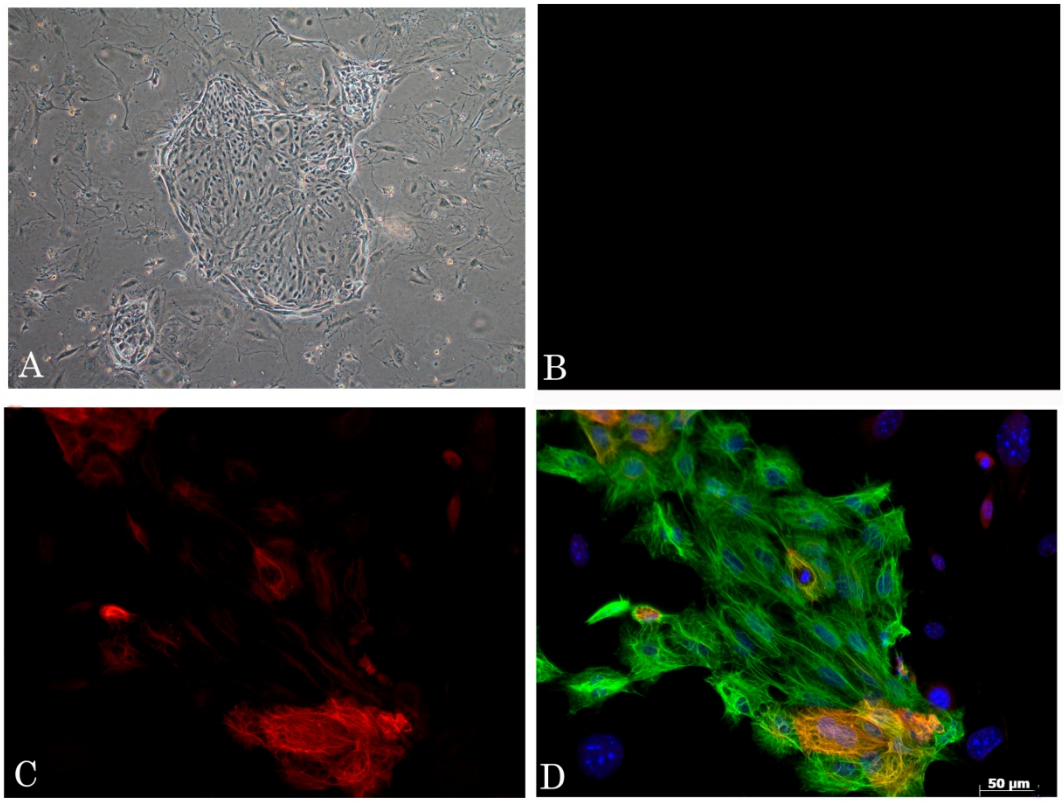

Figure 28 Morphology and expression of epidermal markers in colonies at day 14 of the direct co-culture protocol.
A: At day 14 elevated cells can be seen forming a meshwork-type pattern on top of inactivated PA6 stromal cells; **B:** No primary antibody negative control; **C:** Keratin 5 expression (red) is occasionally detectable within the differentiating cell population; **D:** The majority of the cells (>80%) is strongly positive for keratin 18 staining (green); yellow staining indicates an overlap in keratin 18 (green) and keratin 5 (red) expression. The surrounding PA6 cells are identified by DAPI (blue) staining. Scale bars=50µm.

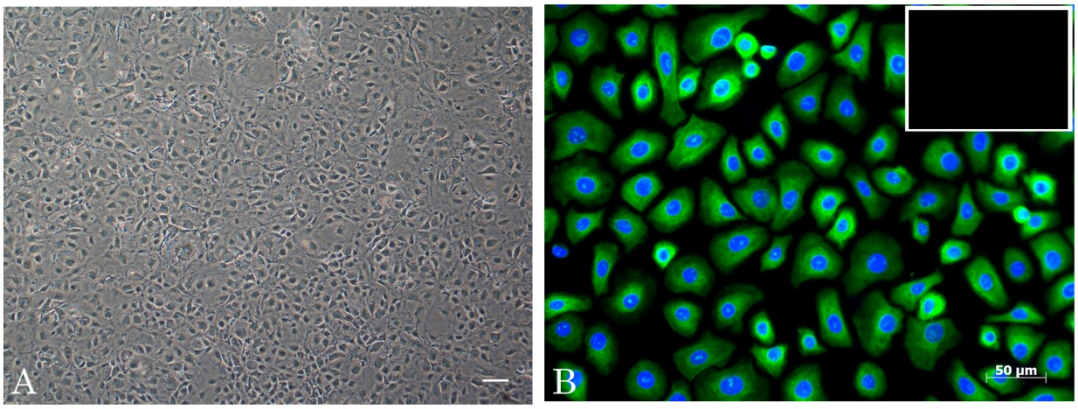

Figure 29 Homogenous population of keratin 14 positive pKPCs obtained at day 21 of the direct co-culture differentiation protocol.
A: Morphology of pKPCS showing cells of similar size and shape; **B:** Immunocytochemistry of pKPCs shows that most cells stain brightly positive for keratin 14 expression. Insert shows a no primary antibody negative control. Scale bars= 50µm.

5.3.2 Growth factors within PA6 CM enable KCL-002 differentiation into early epidermal precursors

5.3.2.1 Expression of epidermal markers

In order to assess the ability of growth factors within PA6 CM to induce epidermal differentiation from hESCs, the presence of epidermal proteins was examined. When PA6 CM and type IV collagen were used as inducers of epidermal differentiation, similar results were obtained to the direct co-culture method. At day 4 of the differentiation protocol, the majority of the cells are positive for simple epithelial keratin 18 and residual nuclear Sox-2 expression was observed by immunofluorescence microscopy (Figure 30). At day 7, no Sox-2 expression was detected and the number of keratin 18 positive cells had decreased significantly. In addition, a few cells expressing basal keratin 14 could be detected (Figure 30). By day 14, small colonies expressing keratin 14 began to form with some colonies still expressing

keratin 18 (Figure 31). Most cells at this stage were also positive for α6-integrin expression. Following the rapid adhesion enrichment approach, only cells expressing high levels of ß1-integrin remained (Figure 32). These cells were also positive for keratin 14 and formed small colonies of highly proliferative cells. When cultured in DKSFM, these colonies expanded into epithelial-like colonies which stained positive for keratin 14. However, after 2-3 passages, a high number of binucleated cells with altered structures and accumulation of autophagic vacuoles within cytoplasm was seen, indicating early cellular senescence (Figure 33). Consequently, the cells could not be expanded past this point. Therefore, an alternative approach relying on indirect co-culture was exploited.

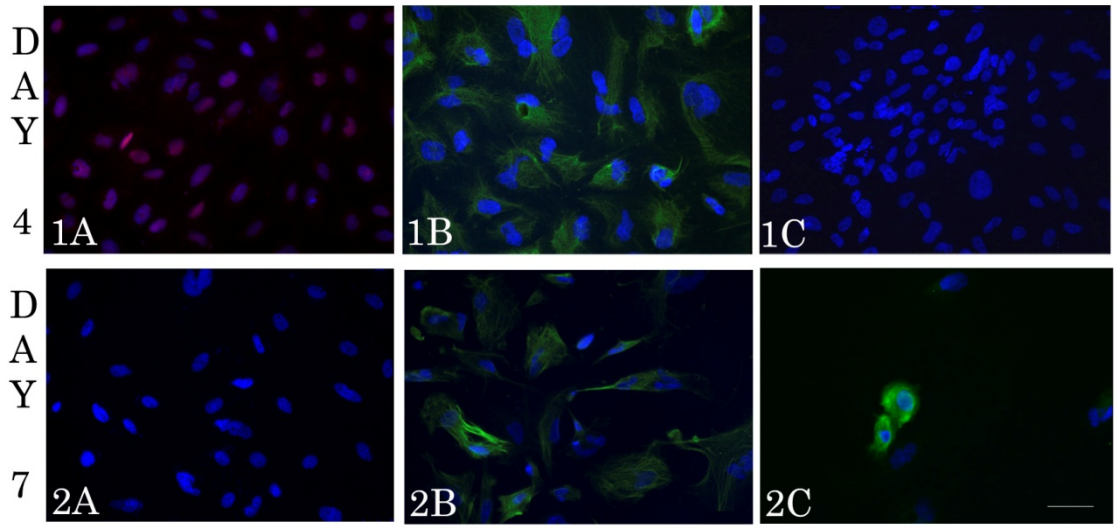

Figure 30 Expression of epidermal differentiation markers in cells treated with PA6 CM at different time points of differentiation
1: Day 4; **2:** Day 7.
A: Sox-2; **B:** Keratin 18; **C:** Keratin 14. At day 4, Sox2 expression can still be detected (1A), cca 80% of cells stain positive for keratin 18 (1B), while no keratin 14 expression could be detected (1C). At day 7, no Sox-2 positive cells were seen (2A) and the percentage of keratin 18 postive cells has decreased to cca 50% (2B), while a few cells expressing keratin 14 could be detected (2C). Scale bar=50μm

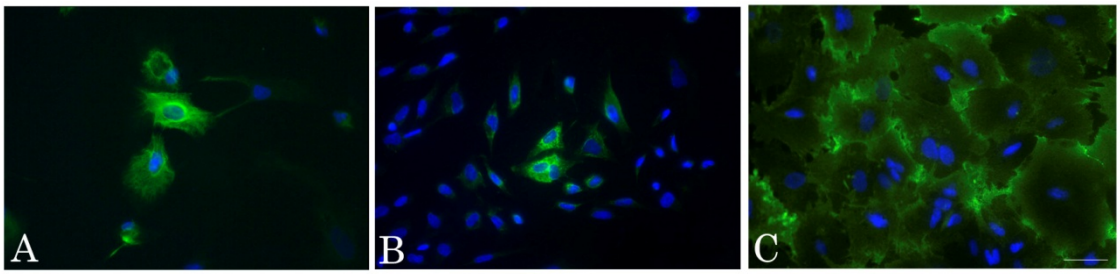

Figure 31 Expression of epidermal differentiation markers in cells treated with PA6 CM at day 14 of differentiation protocol.
A: Residual keratin 18 positivity could be detected in cca 10% of cells; **B:** Small colonies positive for keratin 14 protein begin to appear; **C:** Most cells (>80%) express α6-integrin. Scale bar=50μm.

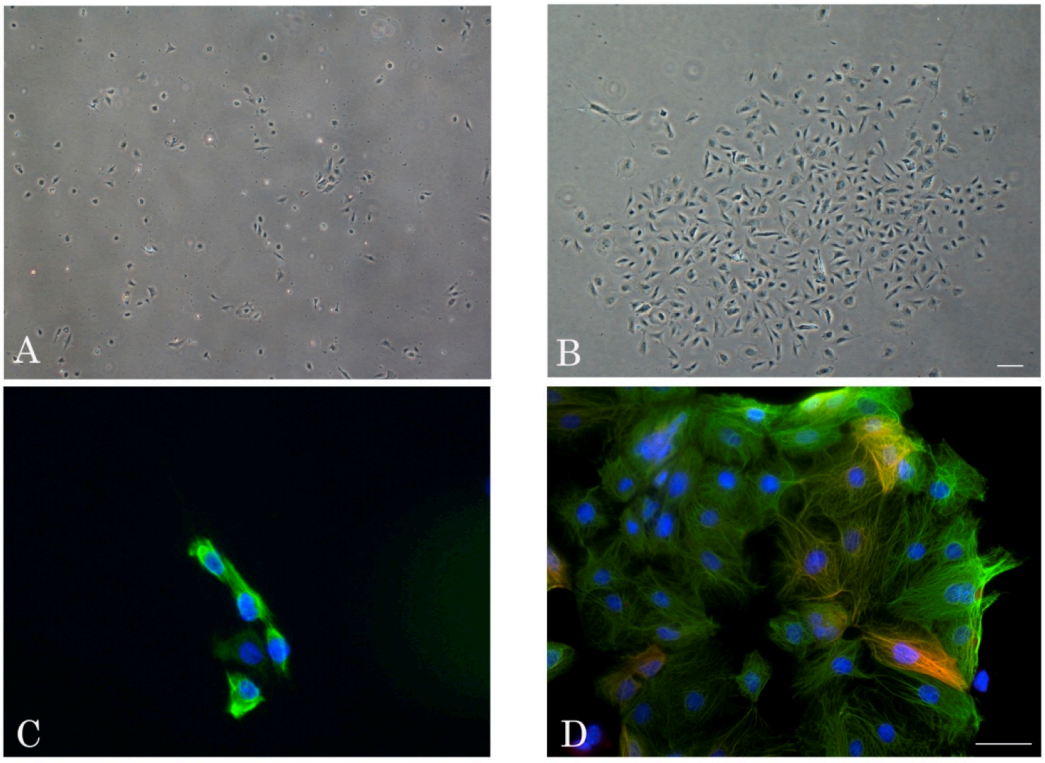

Figure 32 Morphology and expression of epidermal differentiation markers in a population of expanded pKPCs.
A: Following the enrichment procedure, only rapidly adhering cells remain; **B:** These cells grow into much larger colonies; **C:** Rapidly-adhering cells form small colonies positive for keratin 14 (green); **D:** The resultant colonies are homogenously positive for keratin 14 (green) with some keratin 18 (red) expression. Scale bar = 100 μm in A and B, and 50μm. in C and D.

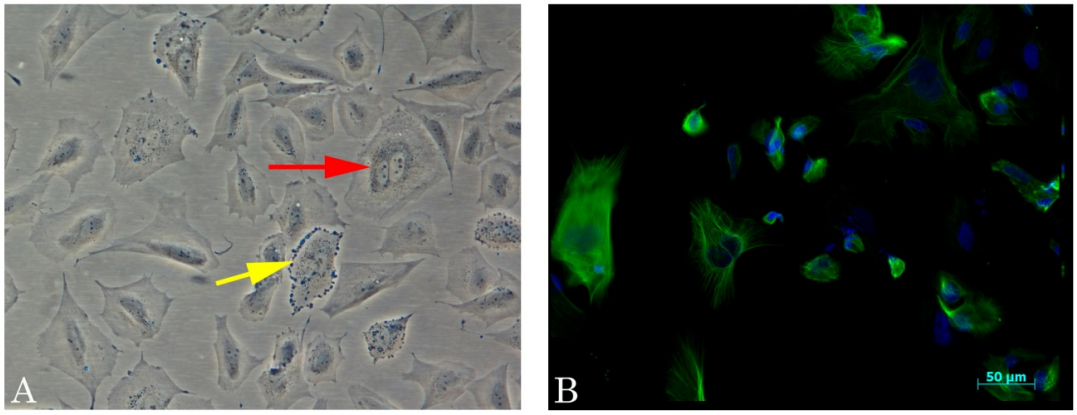

Figure 33 Cellular senescence within the population of pKPCs obtained in the differentiation protocol using PA6 CM.
A: Severely altered morphology, accumulation of autophagic vacuoles (yellow arrow) and the presence of binucleated cells (red arrow) are indicative of early cellular senescence; **B:** Immunofluorescence analysis shows highly dysmorphic heterogenous keratin 14-positive pKPCs (green). Scale bar=50µm.

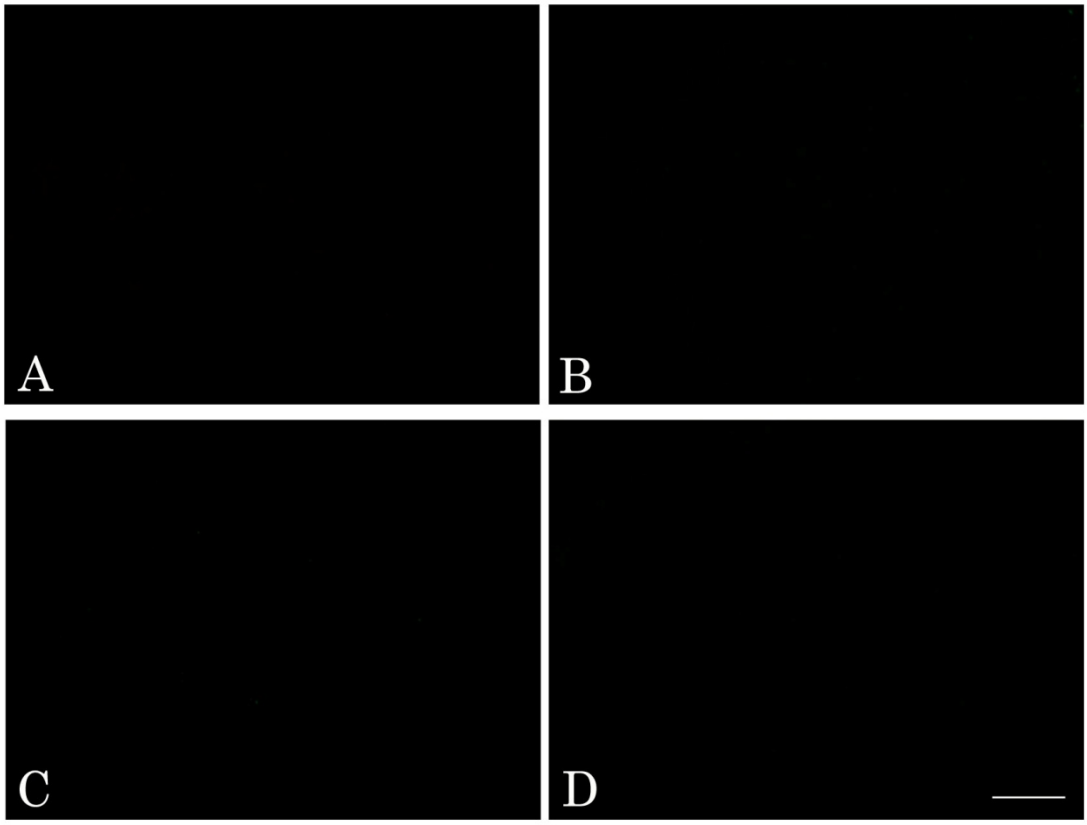

Figure 34 Representative images of no primary antibody negative controls
A: Sox2; **B:** keratin 18; **C:** α6-integrin; **D:** keratin 14. Scale bar=50µm.

5.3.3 Indirect co-culture protocol (Transwell Systems) supports efficient differentiation of KCL-002 towards epidermal cell lineages

5.3.3.1 Morphology and expression of epidermal differentiation markers

The efficiency of the indirect co-culture method was initially confirmed by morphology of the differentiating cells and immunofluorescence analysis for epidermal marker proteins. The cells displayed similar morphological characteristics as in the direct co-culture protocol (Figure 35). By day 4, colonies begin to flatten and a meshwork pattern of elevated cells was seen at day 7. When subcultured on type IV collagen, rapidly-adhering cells were seen forming uniform colonies which underwent further expansion. Large sheets of pKPCs positive for basal keratin 14 formed at day 14 as indicated by immunofluorescence analysis (Figure 36). However, these cells still maintained expression of simple epithelial keratin 18. Some holoclone-like colonies could be observed in those further cultures, which expressed cytoplasmic keratin 14 and nuclear transcription factor p63 (Figure 37). These pKPCs could be further expanded up to 5 passages while maintaining keratin 14 expression. In order to compare the efficiency of epidermal differentiation between the two protocols (PA6 CM vs. indirect co-culture) quantification of immunofluorescence data was carried out.

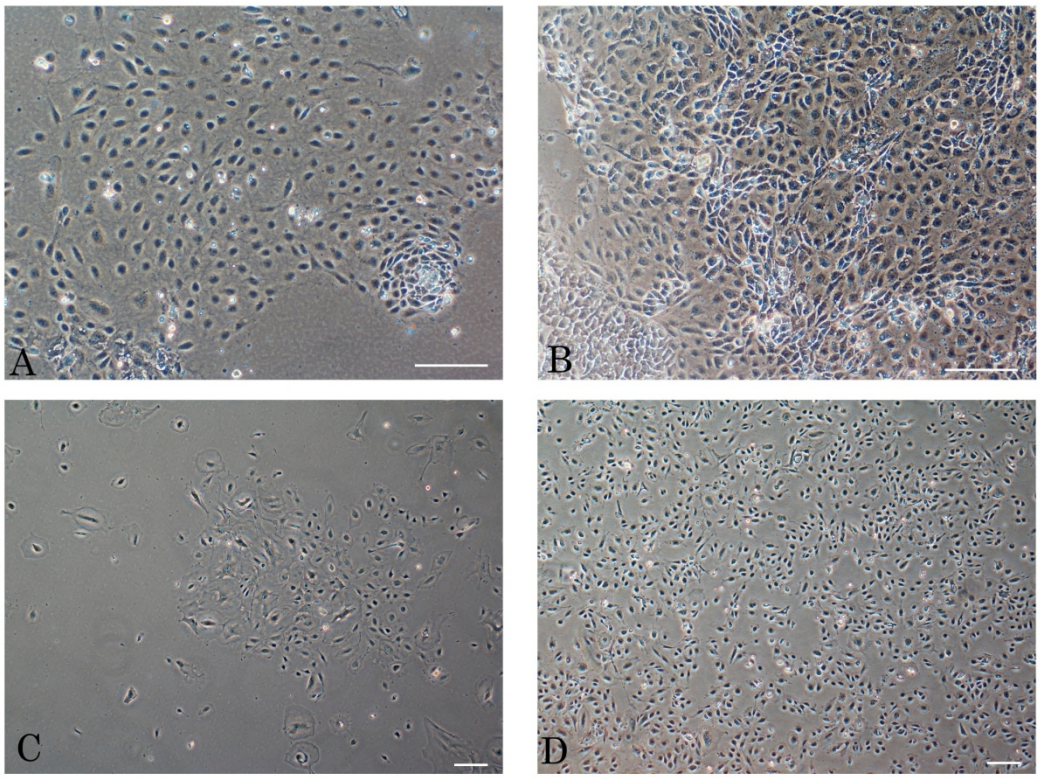

Figure 35 Morphology of differentiating hESC colonies in the indirect co-culture method.
A: Day 4- Colonies begin to flatten and elevated cells can be seen; **B:** Day 7- Meshwork-type pattern of elevated cells is formed; **C:** Day 14- Following the enrichment procedure, rapidly-adhering cells form colonies composed of mostly round cells; **D:** Day 21- Large colonies of homogenously appearing cells can be seen. Scale bars=100µm.

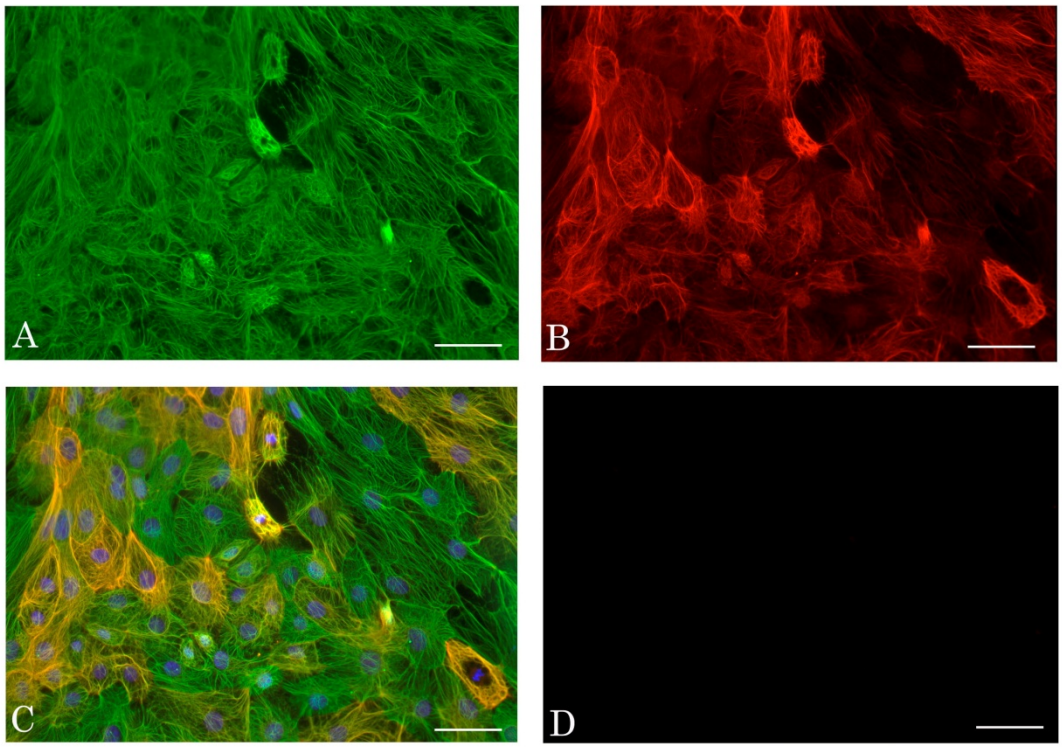

Figure 36 Expression of epidermal markers keratins 14 and 18 at day 14 of differentiation in the indirect co-culture protocol.
A: Keratin 14 (green); **B:** Keratin 18 (red); **C:** Merged image; **D:** No primary antibody negative control. Scale bar=50µm.

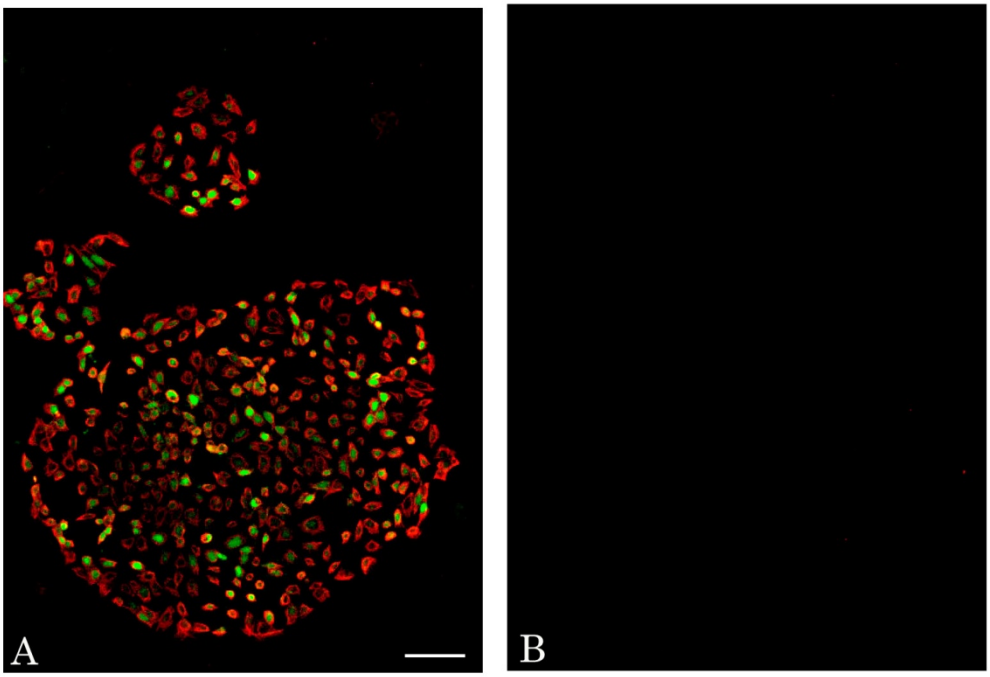

Figure 37 Some holoclone-like colonies can be detected by immunocytochemistry at day 18.
A: These colonies stain positively for keratin 14 (red) and p63 (green); B: No primary antibody negative control. Scale bar=100µm

5.3.3.2 Quantification of immunofluorescence data and statistical analysis

Quantification of immunofluorescence positivity for keratin 14 protein expression was carried out to compare the efficiency of epidermal differentiation in PA6 CM vs. indirect co-culture methods (Figure 38). By Day 14, about 45% more keratin 14-positive cells were detected in indirect co-culture vs. the PA6 CM protocol (65% vs. 19%). By Day 21, approximately 15% more keratin 14-positive cells were observed in indirect co-culture method compared to PA6 CM protocol (77.5% vs. 62%). Statistical analysis confirmed a significant difference between the efficiency of production of keratin 14 positive cells at both time points. Unpaired two-tailed Students' t-test gave the values p=0.0002 and p=0.0172 at days 14 and 21, respectively. These data suggest a high efficiency for the indirect co-culture differentiation protocol and therefore, epidermal marker changes in the time course of this protocol were further investigated by flow cytometry and quantitative PCR.

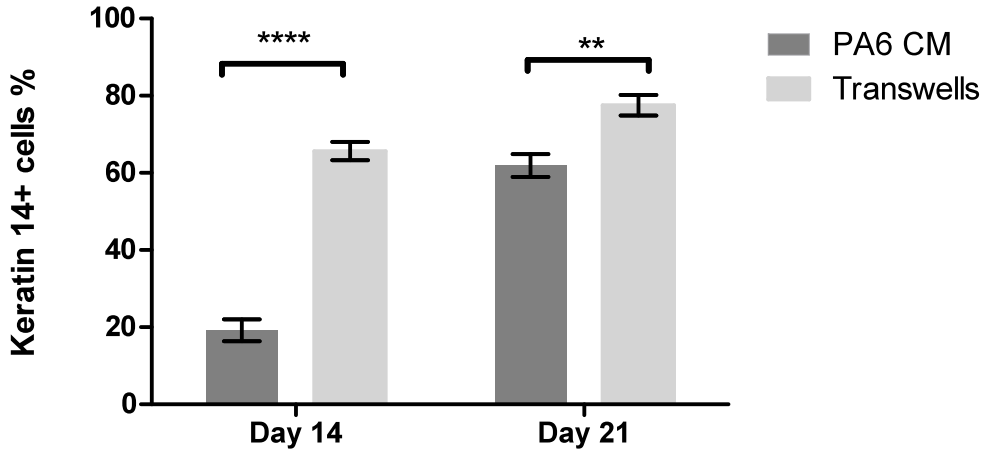

Figure 38 Comparison of PA6 CM vs. indirect co-culture protocol by immunofluorescence staining.
Percentage of keratin 14-positive cells as calculated per 3 independent fields of 100 DAPI-positive cells. n=3. Values expressed as mean ± SEM. A significant difference in keratin 14 protein expression was observed when the two differentiation methods were compared. Unpaired two-tailed Students' t-test
**** $p<0.0001$, ** $p<0.01$

5.3.3.3 Keratin 14 expression changes during indirect co-culture differentiation

In order to validate the presence of keratin 14 protein in the differentiating cell population, flow cytometry analysis was performed at Day 0 (undifferentiated hESCs negative control), 14, 21 and 30 of differentiation. This showed a progressive increase in keratin 14 expression (Figure 39). At Day 15, 55.59% were positive for keratin 14, while at days 25 and 30 the number of positive cells increased to 65.36 and 70.52%, respectively. It is worth noting, however, that keratin 14 expression at day 30 of differentiation still has not reached the same level as that in a positive control sample (NHKs) (Figure 39E), suggesting that not all the cells have obtained a basal epidermal phenotype. Nevertheless, the results obtained by

flow cytometry are in agreement with the data from the immunofluorescence analysis. The next step was to confirm the presence of epidermal markers at the mRNA level.

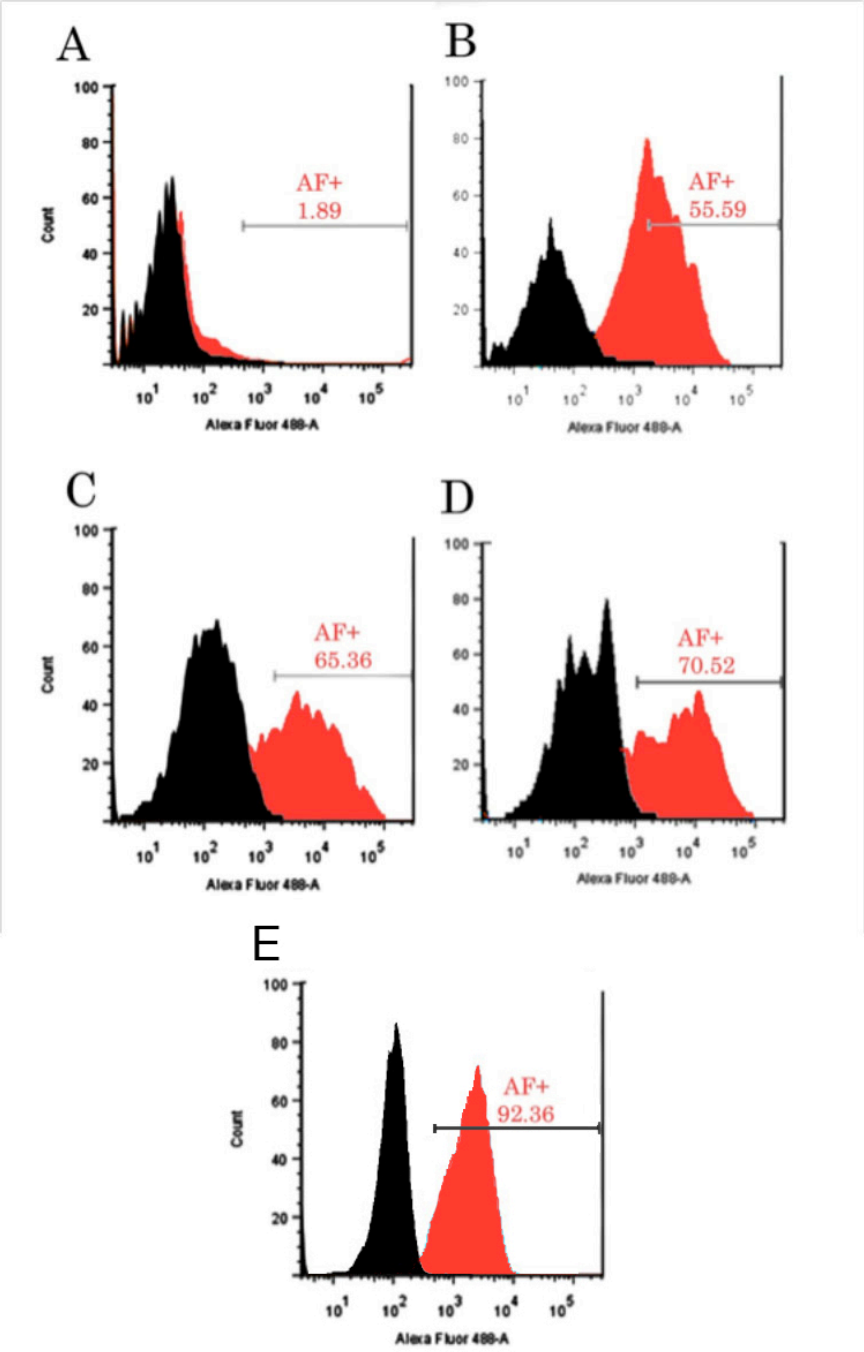

Figure 39 Flow cytometry for keratin 14 expression during the course of differentiation in the indirect co-culture protocol.
Vertical axis: Cell count. Horizontal axis: Alexa Fluor 488 intensity. **A:** Day 0 (negative control, undifferentiated hESCs); **B:** Day 14; **C:** Day 21; **D:** Day 30; **E:** Positive control (NHKs).

5.3.3.4 Quantitative PCR results

Quantitative PCR was performed at Day 7, 14 and 30 of differentiation in order to monitor efficiency of the differentiation protocol (Figure 40). Nanog was used to monitor the presence of undifferentiated hESCs, keratin 18 was used as a marker of early ectodermal differentiation, keratin 14 gene expression was assessed as a marker of a more mature epidermal fate, and involucrin as a marker of terminal differentiation. Gene expression for keratin 14 increased 17.03 fold at day 14 and 190.71 fold at day 30 when compared to undifferentiated hESCs. Keratin 18 expression increased 13.63 fold by day 7 days and then went down progressively to 7.40 and 4.19 at days 14 and 30, respectively. In addition, some involucrin gene expression could be detected at day 30 (3.72 fold increase as compared to undifferentiated hESCs). Expression of the pluripotency gene Nanog went down by 0.527 at day 7 and disappeared completely in the later stages of differentiation.

5.3.3.5 Population doubling assay

A population doubling assay was carried out to establish the number of divisions that pKPCs were capable of undergoing before reaching senescence. The assay revealed that pKPCs could undergo approximately 9 population doublings before reaching senescence (Figure 41). An approximate population doubling time was 4 days.

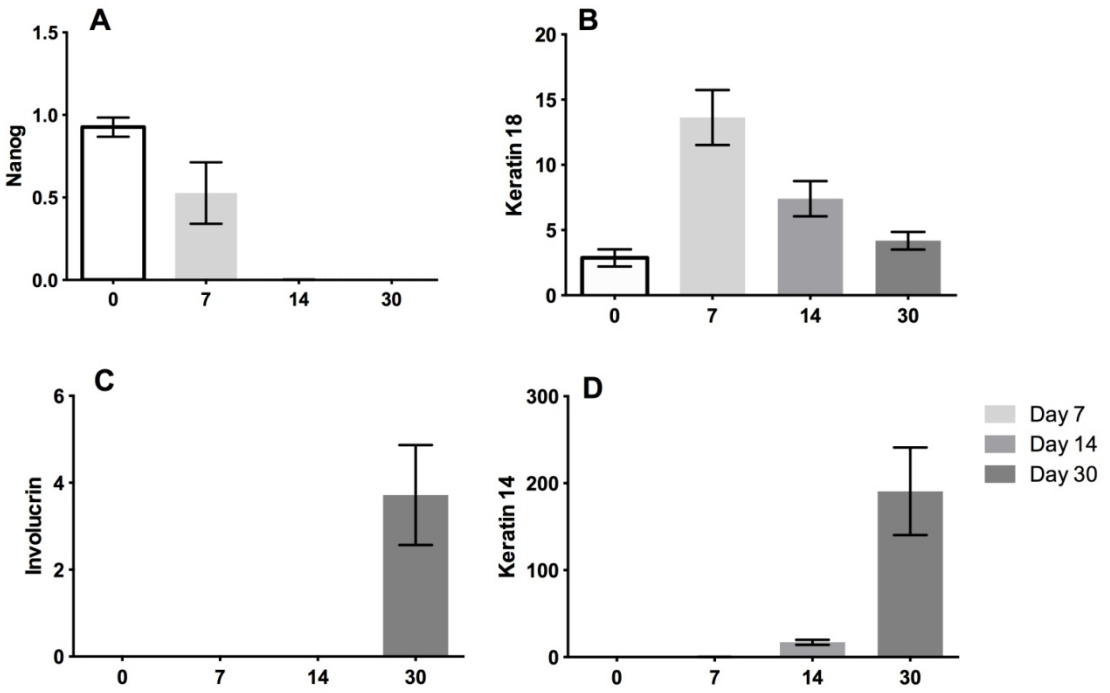

Figure 40 Gene expression changes during the course of differentiation in the indirect co-culture protocol.
The graphs show gene expression ratios for 4 genes of interest at 3 different time points as normalized to the appropriate gene expression ratio in undifferentiated hESCs. Expression of each gene was compared to housekeeping (GAPDH) gene expression. **A:** Nanog; **B:** keratin 18; **C:** involucrin; **D:** keratin 14. Values are expressed as mean±SD. n=3.

Figure 41 Population doublings of pKPCs obtained in the indirect co-culture differentiation protocol plotted against the number of days in culture.
Only 9 population doublings were observed before the cells entered senescence. Approximate population doubling time was 4 days.

5.3.3.6 Technical discussion

A potential technical limitation of the differentiation study using PA6 stromal cell line co-culture is the fact that pKPCs obtained were cultured under feeder-free conditions in the presence of DKSFM. It is pausible that these conditions were suboptimal for further expansion of epidermal progenitors. Therefore, an alternative method of culture on inactivated 3T3 fibroblasts feeder-layer, as described by Rheinwald and Green, could have been employed to improve proliferation capacity and expand the lifespan of pKPCs.

5.4 DISCUSSION

The results described in this set of experiments indicate that both direct co-culture with PA6 cells and the growth factors secreted by the stromal cells can induce epidermal commitment of hESCs. The most efficient differentiation was achieved under indirect co-culture conditions with over 75% of differentiated progeny expressing the basal keratin 14 marker. Therefore, the data demonstrated that SDIA promoted by mesenchymal stromal feeders and type IV collagen is able to mimic the effects of embryonic mesodermal stimulation and direct hESCs differentiation towards keratinocyte lineages *in vitro*.

The first suggestion that SDIA promoted by stromal cell line could direct differentiation of ESCs towards epidermal fate came as a side result of a study aimed at inducing dopaminergic differentiation from ESCs (Kawasaki et al., 2000). In that study, the authors showed that BMP-4 treatment could

successfully divert ectodermal differentiation promoted by SDIA to non-neural ectoderm, resulting in expression of the epidermal markers E-cadherin and keratin 14.

5.4.1 Direct co-culture with PA6 stromal cells for inducing epidermal differentiation of hESCs

The direct co-culture method has previously been successfully exploited to derive a homogenous population of ectodermal precursors from hESCs (Aberdam et al., 2008). In this approach, a stable somatic ectodermal cell population that was 100% positive for keratin 18 was obtained. The authors suggested that derivation of such a primitive ectodermal population is particularly useful for designing *in vitro* models to recapitulate early embryonic epidermogenesis and lineage specification and can also serve as a potential source for epidermal stem cells. However, lentiviral transduction with ΔNp63 construct was required to induce the production of putative epidermal progenitors, limiting the potential applicability for clinical therapy.

More importantly, significant levels of mRNA for simple epithelial keratins have been well documented in hESCs (Bhattacharya et al., 2004, Liu et al., 2006a, Cai et al., 2006, Schwartz et al., 2005). A meta analysis of hESC transcriptome (http://amazonia.montp.inserm.fr/) initially suggested that simple epithelial keratins are under-expressed in undifferentiated hESCs (Assou et al., 2007). However, later this finding was explained by the fact that during spontaneous differentiation large differentiated cells, typically

found at the borders of the colonies accumulate increased amount of keratins 8 and 18 (Maurer et al., 2008). The expression of keratins 8 and 18 has been suggested to reflect the epithelial nature of undifferentiated hESCs and explain various characteristics of hESCs, such as the flattened epithelial phenotype of colonies (Maurer et al., 2008). Therefore, the overall efficiency of epidermal differentiation from hESCs cannot be accurately assessed by keratin 18 expression alone.

Therefore, the first aim of my set of experiments was to adapt and replicate the direct co-culture method for the KCL-002 hESC line and evaluate the efficiency of epidermal differentiation by expression of basal keratin 14 as opposed to simple epithelial keratin 18.

First, the existing protocol (Aberdam et al., 2008) had to be optimized for the KCL-002 hESC line, and morphological observations suggested mitomycin C treatment of PA6 cells was the method of choice for direct co-culture with KCL-002 hESCs. Mitomycin C inactivation appeared to preserve viability of hESCs, while still enabling SDIA promoted by PA6 cells. This observation is in agreement with previous reports on the effects of mitomycin C on SDIA (Vazin et al., 2008). When expression of the basal epidermal keratin 14 protein was evaluated by immunocytochemistry, a homogenous population of pKPCs was observed. Therefore, direct co-culture with mitomycin C-inactivated PA6 cells was able to induce efficient epidermal differentiation of the KCL-002 hESC line.

However, despite producing keratin 14-positive cells, the PA6 co-culture method has some drawbacks. The main disadvantage of a direct co-culture

method is that it makes it difficult to isolate the potential target cells while maintaining their homogeneity. This in turn makes full characterization challenging. Furthermore, direct co-culture with animal cells renders any clinical application impossible and a feeder-free system would presumably be required for use in any human cell-based therapy.

5.4.2 Conditioned medium enriched in PA6-secreted growth factors in promoting epidermal differentiation of hESCs

Given the methodological concerns outlined above, the use of PA6 CM enriched in soluble growth factors secreted by stromal cells could offer a good alternative if efficient differentiation was achieved. Moreover, previous reports have suggested that PA6 CM might indeed promote the same level of SDIA as intact PA6 cells (Schwartz et al., 2005, Morizane et al., 2006, Vazin et al., 2009, Vazin et al., 2008, Swistowska et al., 2010). With regards to epidermal differentiation, growth factors secreted by the PA6 stromal cells, in combination with type IV collagen as substrate, were examined for their potential to induce epidermal differentiation from hESCs. As evaluated by immunocytochemisty, differentiating cells begin to express minimal amounts of keratin 14 protein by day 7, while by day 14 approximately 20% of differentiating colonies expressed keratin 14 along with keratin 18 expression. This suggests that the ectodermal progenitors obtained at this stage have started to acquire a more defined epidermal fate. The cells enriched for β1-integrin expression continued to proliferate and by day 21 formed large epithelial-like colonies, almost two-third of which were

positive for keratin 14 expression. However, these pKPCs could only be maintained for up to 2-3 passages, after which the cells started showing signs of early senescence. Furthermore, these cells could not be expanded making any potential application impossible.

Therefore, even though soluble growth factors within PA6 CM appear to be sufficient to induce some epidermal differentiation and production of keratin 14-positive cells, the putative paracrine effects are insufficient for generation of a functional keratinocyte from hESCs. It is very likely that a lack of cellular signalling and/or incorrect temporal exposure to growth factors hinders the differentiation of highly proliferative precursors.

5.4.3 Indirect co-culture method using permeable support Transwell systems as an inducer of epidermal differentiation from hESCs

As an alternative method, indirect co-culture that relies on the use of permeable Transwell support Systems was evaluated for its potential to induce differentiation of hESCs towards epidermal lineages. Transwell Systems provide a more suitable microenvironment for differentiation as well as more accurate temporal induction with the secreted growth factors. During the course of differentiation the cells progressively acquire epithelial-like morphology. By day 7, undifferentiated colonies change morphology into much flatter and more spread-out looking cells. After a further 7 days elevated cells can be seen forming a meshwork-type pattern. When these cells were enriched based on rapid adherence to type IV

collagen and subcultured in DKSFM, the expanded population was mainly composed of round cells.

Immunofluorescence analysis indicated that this approach allows for large keratin 14 and 5-positive epithelial sheets to be formed (77.5% positive for keratin 14). These sheets could be further subcultured on type IV collagen substrate and form holoclone-like colonies with some p63-positive cells, indicating the presence of highly proliferative epidermal progenitors.

As discussed in Chapter 1, in order to achieve efficient epidermal differentiation of hESCs, it is vital to closely replicate epidermal morphogenesis. *In vivo* well-defined changes in gene expression accompany epidermal morphogenesis and stratification (Fuchs and Green, 1980). Therefore, gene expression during the course of differentiation was monitored by quantitative PCR and appeared to closely mimic epidermal morphogenesis i*n vivo.* The expression of the keratin 18 gene, a marker for early ectoderm, increases during the first stages of differentiation, suggesting that hESCs have successfully acquired ectodermal fate, and then decreases steadily indicating that the cells have obtained a more defined epidermal fate. At the same time, the expression of the keratin 14 gene shows a steady increase and by day 30 it is increased 190-fold compared to undifferentiated hESCs. A progressive increase in keratin 14 expression was also confirmed by flow cytometry. However, flow cytometry data indicated that keratin 14 protein expression does not reach the same level as in human primary keratinocyte culture (70.5% in pKPCs versus 92.36% in primary keratinocytes). The expression of the involucrin gene at day 30

indicates that pKPCs have the capacity to undergo terminal differentiation. However, this expression could not be detected by immunocytochemistry suggesting that despite gene expression the monolayer culture prevents the protein from being assembled properly (Banks-Schlegel and Green, 1981). It has been suggested previously that in monolayer cultures, involucrin is only synthesized by large cells which would preferentially adopt a suprabasal position in response to stratification (Watt and Green, 1982).

The pKPCs purified on the basis of high surface expression of ß1-integrin, and as a result of that rapid adhesion to type IV collagen, were capable of forming actively growing colonies. This is in agreement with data obtained for NHKs enriched in putative epidermal stem cells *in vitro* (Dong et al., 2007, Jones and Watt, 1993, Li et al., 1998). As discussed previously, an epidermal stem cell is defined by two fundamental qualities: extensive capacity for self-renewal and the ability to generate terminally differentiated cells. The pKPCs obtained in this differentiation system fulfilled both requirements for epidermal stem cells, since they formed actively growing colonies that contained, in addition to rapidly adhering cells, slowly adhering and involucrin-positive cells. Furthermore, the increased number of viable cells compared to direct co-culture and PA6 CM methods shows that indirect Transwell co-culture methodology provides a milieu that maintains cells adequately.

Overall, the indirect co-culture method using Transwell inserts was significantly more efficient than PA6 CM alone in directing hESCs towards epidermal fate. The pKPCs produced under these differentiation conditions

displayed increased survival and proliferation potential as well as higher levels of keratin 14 protein. The efficiency of the indirect co-culture method in enabling hESCs differentiation towards epidermal lineages suggests the existence of co-ordinated signalling between the two cell types, which stimulates their normal growth and differentiation, as might be observed *in vivo*.

5.4.4 Drawbacks of the indirect co-culture method

However, despite the effectiveness of this method, pKPCs still exhibited a lower expansion potential than primary cultures: only 9 population doublings were observed before the cells became senescent. Several previous differentiation studies have reported a limited proliferation potential of hESC-derived keratinocytes with less than 15 population doublings (Aberdam et al., 2007, Green et al., 2003, Ji et al., 2006, Metallo et al., 2008b). Normal human primary keratinocyte are known to undergo what is referred to as "p16 senescence," which occurs abruptly and with ever increasing frequency during serial passage (Dickson et al., 2000, Rheinwald et al., 2002). It is well known that keratinocyte lifespan is determined by the timing of derepression of the *CDKN2A* gene, encoding the cell cycle inhibitors $p16^{INK4A}$ and $p14^{ARF}$. Therefore, in order to become immortalized, primary keratinocytes must undergo mutations or be engineered to evade this mechanism by telomerase catalytic subunit (TERT) expression (Kiyono et al., 1998, Dickson et al., 2000, Rheinwald et al., 2002). However, it has been shown that hESC-derived keratinocytes cannot in fact be immortalized

in that way (Iuchi et al., 2006). This study was followed by a comprehensive analysis of three hESC-derived keratinocyte lines for morphology, marker expression, growth requirements, motility, and histogenic potential (Dabelsteen et al., 2009). The results suggested that despite sharing many features with human primary keratinocytes, hESC-derived epidermal cells display early $p16^{INK4A}$, as well as hypermotility and incomplete differentiation properties, suggesting that these cells represent an incomplete or abnormal form of p63-positive lineage development. It is plausible that the lack of *in vivo* signalling hinders the differentiation process and leads to the production of seemingly abnormal phenotypes.

Furthermore, even though the indirect co-culture method avoids any direct contact between the two cell types, it still relies on ill-defined animal-based components to induce epidermal differentiation from hESCs. Potential immunogenic factors such as sialic acid or Neu5Gc (Martin et al., 2005, Sakamoto et al., 2007) and animal pathogens are still able to pass through the pore of Transwell inserts and contaminate the resulting culture. Therefore, a defined and xeno-free epidermal differentiation method would be required to comply with GMP reluations for clinical applications.

5.4.5 Divergent effects of PA6 stromal cells on ESCs differentiation

Recently, mouse ESCs co-cultured with PA6 cells in the presence of BMP-4 and retinoic acid were shown to undergo mesodermal differentiation with over 90% colonies robustly expressing desmin and nestin, both markers of

mesodermal lineages (Torres et al., 2012). The authors have suggested that BMP-4 activation of Smad-dependent genes in ESCs can be modulated by retinoic acid or co-culture with PA6 stromal cells to direct recruitment of activated Smads to specific master genes that control the mesodermal gene expression program. Interestingly, the authors reported that they could not detect any epithelial cell commitment (Torres et al., 2012).

The differences in results described in this chapter and those by Torres *et al.* could be explained by intricate differences of methods that could influence the fine-tuning of Smad-dependent gene expression, thereby influencing stem cell fate. In this context, retinoic acid signalling was previously reported to control the duration of BMP-4-triggered phosphorylation of Smad1/5/8 (Sheng et al., 2010). It has been reported that retinoic acid stimulation of ES/PA6 co-cultures induced efficient differentiation of ESCs into spinal cord cell progenitors (Wichterle et al., 2002). On the other hand, retinoic acid stimulation was shown to induce the differentiation of ESCs into mesenchymal and skeletal muscle cells when the cells are differentiated as embryoid bodies (Kawaguchi et al., 2005, Kennedy et al., 2009). Similarly, BMP-4 was also reported to synergise with Activin A to induce the formation of mesendoderm (Vallier et al., 2009). Therefore, it is possible that the choice of a particular cell fate is controlled by cross-talk between BMP-4-, retinoic acid- and SDIA-activated signalling pathways and depends on both the concentration and temporal arrangement of these factors. Accordingly, a recent study, which described derivation of functional melanocytes from hESCs using BMP-4 and ascorbic acid treatment, also evaluated the effect of varying concentrations of BMP-4 on

the engagement of hESCs at the earliest stage of cell commitment (Nissan et al., 2011). It was shown that the highest concentration (5–0.5μM) directed the towards epithelial lineages, while intermediate (0.02–0.004μM) and the lowest (0.004-0.0008μM) concentrations led to generation of neural crest and neural cells, respectively. In support of this observation, the study described in this chapter showed that when high concentration of BMP-4 was applied during the earliest stage of differentiation induction, hESCs could be directed towards epithelial cell lineages and produce Keratin 14 positive epidermal progenitors.

5.4.6 Conclusion and future directions

The results described in this chapter demonstrate that the sequential contribution of mesenchymal inducers can replicate *in vitro* the effects of embryonic mesodermal stimulation and encourage epidermal differentiation from hESCs. SDIA promoted by growth factors secreted by PA6 cells can direct hESCs towards ectodermal lineages, while BMP-4 is able to antagonize neural fate, leading to development of non-neural ectoderm. Subsequent induction with type IV collagen and appropriate culture medium encourages further differentiation to epidermal precursors.

Despite the efficiency of this method, several drawbacks hinder its clinical potential. Therefore, alternative methods should be exploited. Such methods should be able to replicate normal epidermal morphogenesis by mimicking the natural microenvironment which supports keratinocyte development.

CHAPTER 6 NATIVE DE-CELLULARIZED ECM AND COMBINED SYNERGETIC EFFECTS OF BMP-4 AND RETINOIC ACID TO DIRECT EPIDERMAL DIFFERENTIATION OF hESCs

HYPOTHESIS:

Extracellular matrix produced by normal human dermal fibroblasts has the potential to serve as an efficient inducer of epidermal differentiation by mimicking the natural keratinocyte environment, i.e. basement membrane of human skin, in combination with appropriate differentiation conditions and induction medium.

AIMS:

1. Optimize the protocol for reproducible ECM production from HDFs and characterize the resulting matrix by immunofluorescence micriscopy.
2. Adapt KCL-002 hESCs to grow on native de-cellularized ECM produced by HDFs and assess the expression of pluripotency-associated proteins by immunofluorescence.
3. Devise tissue culture conditions necessary to promote differentiation of pKPCs from undifferentiated hESCs; analyse the expression of

marker proteins to confirm progression of differentiation by immunofluorescence and flow cytometry and investigate the colony forming efficiency and proliferation of pKPCs produced.

4. Compare the efficiency of HDF-ECM protocol to that of PA6 protocols, as described in Chapter 4.

6.1 INTRODUCTION

6.1.1. ECM as a niche for adult skin stem cells

Adult stem cells, including the ones found in skin, reside within tissue-specific niches, which maintain their stem cell fate (Scadden 2006; Morrison 2008). As discussed previously, in epidermis, resident stem cells adhere to a specialized sheet-like form of the ECM, the BM. The components of BM function to maintain skin homeostasis by balancing the proportion of quiescent and actively proliferating cells. ECM is the non-cellular component present within all tissues and organs, which provides not only essential physical scaffolding for the cellular constituents but also plays a crucial role in tissue morphogenesis, differentiation and homeostasis. Although, fundamentally, the ECM is composed of the same constituents (i.e. water, proteins and polysaccharides), each tissue has an ECM with a unique composition and topology that is acquired during tissue development through dynamic and reciprocal interactions of various cellular components with the evolving microenvironment.

In the skin, fibroblasts secrete and organize the ECM proteins, which provide tissue organization and structural support for keratinocytes, regulating cellular functions such as adhesion, migration, growth and survival (Buck and Horwitz, 1987, Geiger et al., 2001, Hynes, 1999). Skin BM is the fusion of two laminae, basal lamina, composed of the lamina densa and lamina lucida, and lamina fibro-reticularis (Paulsson, 1992). The lamina densa is a lattice-like structure, which is composed mainly of type IV

collagen. The lamina lucida is a region where hemidesmosome complex proteins congregate. It is transvered by anchoring filaments. The proteins and glycoproteins in this region include the α6 and ß4 integrin subunits, type XVII collagen, and laminins (predominantly laminin-332) as well as tetraspanin and nidogen-entactin (Litjens et al., 2006, Margadant et al., 2008). The lamina fibro-reticularis underlies the lamina densa and contains fine collagen fibrils and anchoring fibrils, which are composed mainly of type VII collagen (Sakai et al., 1986, Lunstrum et al., 1986). Fibronectin is also located in the DEJ (Couchman et al., 1990) and promotes adhesion and migration (O'Keefe et al., 1985) while inhibiting terminal differentiation (Adams and Watt, 1989). Basal keratinocytes are anchored to BM via integrins which bind ECM components via their extracellular domains: α2ß1 heterdimer is a collagen receptor, α3ß1 and α6ß4 predominantly bind laminin and αvß5 binds vitronectin (Watt and Hertle, 1994, Adams and Watt, 1991). Furthermore, there is a marked variation in ECM and integrin expression within different regions of the skin as well as at different developmental stages (Watt, 2002, Wilhelmsen et al., 2006, Sugawara et al., 2008, Breitkreutz et al., 2009). This variation in turn creates distinct microenvironments that play a role in modulating the properties of different cell populations. It is well recognized that ECM creates an important niche for adult stem cells, not just in skin but also in other adult tissues (Hall and Watt, 1989, Scadden, 2006, Spradling et al., 2001, Watt and Hogan, 2000). As discussed previously, a high level of ß1 integrin expression is a known marker of epidermal stem cells (Jones and Watt, 1993, Jones et al., 1995) and can successfully be used to enrich cell cultures for putative stem cells

(Dong et al., 2007, Jensen et al., 1999). Therefore, there is a growing appreciation that ECM is not inert scaffolding material but an active dynamic structure that plays a vital role in all aspects of skin function and homeostasis.

Considering the importance of ECM in tissue development and maintenance *in vivo,* it is plausible that when provided with an appropriate environment that closely mimicks the natural skin stem cells niche, the pluripotent stem cells would successfully be guided towards epidermal differentiation. Clearly, the molecular composition of collagen and other coating matrices cannot fulfil this function in isolation.

6.1.2. Biomolecular and biophysical characteristics of ECM

The ECM is composed of two main classes of macromolecules: proteoglycans (PGs) and fibrous proteins (Jarvelainen et al., 2009, Schaefer and Schaefer, 2010). The main fibrous ECM proteins are collagens, elastins, fibronectins and laminins (Alberts et al., 2007). PGs fill the majority of the extracellular interstitial space within the tissue in the form of a hydrated gel. Collagen is the most abundant fibrous protein within the interstitial ECM. In fact, up to 30% of the whole body mass is constituted from collagens. Within ECM, collagens provide tensile strength, regulate cell adhesion, support chemotaxis and migration, and direct tissue development (Rozario and DeSimone, 2010). Although within a given tissue, collagen fibres are generally a heterogeneous mix of different types, one type of collagen usually predominates. Fibroblasts are known to deposit the matrix

immediately outside their cell membrane, with the synthesized fibre parallel to the major cell axis (De Wever et al., 2008). By exerting tension on the matrix, fibroblasts are able to organize collagen fibrils into sheets and cables, dramatically influencing the alignment of collagen fibres. The collagen fibre alignment, which in turn regulates cellular behaviour, is an important consideration. Fibronectin is closely involved in directing the organization of the interstitial ECM and also has a crucial role in mediating cell attachment and function. Fibronectin can unfold and stretch by cellular traction forces (Smith et al., 2007). This process exposes cryptic integrin binding sites within the fibronectin molecule, which in turn acts as an extracellular mechano-regulator and influences cellular behaviour (Smith et al., 2007). Furthermore, fibronectin is also important for cell migration during development and, in such a capacity, has been implicated in tumour metastasis (Rozario and DeSimone, 2010, Tsang et al., 2010). Thus, the tissue ECM is a highly dynamic entity that continuously undergoes regulated remodelling, whose precise orchestration is crucial to the maintenance of normal function (Egeblad et al., 2010, Kass et al., 2007).

6.1.3 ECM as a mediator of growth factors signalling

ECM acts as a reservoir of growth factors, which can be released as soluble factors to bind their receptors. Extracellular matrix-associated heparan sulphate PGs regulate the interaction of several heparin-binding growth factors with their respective receptors and, consequently, their biological activity (Schlessinger et al., 1995, Jonca et al., 1997). For example, heparan

sulphate PGs can function as an extracellular storage place for the heparin-binding VEGF isoforms (Park et al., 1993). This binding establishes stable gradients of growth factors, and ECM degradation can then release these growth factors from solid to soluble phase. In addition, some growth factors, such as FGF, require binding to ECM (via heparan sulphate cofactor) prior to association with their actual signalling receptor (Mohammadi et al., 2005, Shi and Massague, 2003). Furthermore, growth factors can bind to ECM proteins themselves without the involvement of PGs (Iyer et al., 2007). Both fibronectin and vitronectin bind hepatocyte growth factor leading to enhanced cell migration (Rahman et al., 2005). Laminin contains multiple EGF-like domains, which can be released by proteolysis and, in soluble phase, bind to EGFRs and modulate its signalling (Panayotou et al., 1989). The key Dpp (a BMP homolog) -binding motif was identified in the C-terminal domains of the two Drosophila collagen IV subunits (Wang et al., 2008). ECM can also act as a storage of latent growth factors. The latent TGF-ß–binding proteins interact with a number of matrix components including collagen, fibronectin and fibrillin, thereby incorporating the different TGF-ß isoforms into ECM in latent form (Olofsson et al., 1995, Unsold et al., 2001). This incorporation is necessary for the subsequent effective activation of TGF-ß through degradation of ECM proteins (Gleizes et al., 1997, Rifkin, 2005).

6.1.4 ECM in ageing, fibrosis and cancer

The importance of ECM has been implicated in ageing (Callaghan and Wilhelm, 2008), fibrosis (Schultz and Wysocki, 2009) and tumour formation (Butcher et al., 2009, Levental et al., 2009). For example, ageing tissue is characterized by a thinning of the BM (Callaghan and Wilhelm, 2008). In addition, collagen fibres are inappropriately crosslinked which leads to tissue stiffening (Robins, 2007). This aberrant mechanical state can severely compromise ECM organization, and modify epithelial organization and function, potentially promoting age-related diseases such as cancer (Coppe et al., 2010, Freund et al., 2010, Sprenger et al., 2008).

6.1.5 Commercial preparations of ECM

Considering the importance of the ECM to so many fundamental cellular processes, extensive research has been devoted to developing a commercially viable ECM preparation that could closely mimic its *in vivo* counterpart. Purified preparations or mixtures of ECM proteins have been used to obtain 2D monolayers (Kuschel et al., 2006). To address the issue of ECM rigidity, functionalized polyacrylamide gels crosslinked with reconstituted BM - generated from EHS mouse carcinoma (commercially available as Matrigel™), collagen type I, fibronectin or ECM peptides – has become a standard approach (Johnson et al., 2007, Pelham and Wang, 1997). Yet, none of these methods truly recapitulates the *in vivo* scenario in which ECM has a 3D architecture that can be easily remodelled. Type I collagen has some useful properties and can be combined with reconstituted BM, purified

laminin or fibronectin to reconstitute some of the biological aspects of normal and diseased interstitial ECM (Friess, 1998, Gudjonsson et al., 2002). Moreover, type I collagen readily assembles into a mechanically tense network of fibrils that can be oriented, functionally modified, and enzymatically or chemically crosslinked and stiffened. Nevertheless, collagen gels are quite heterogeneous, and modifying their architecture changes their organization, pore size and ligand concentration, thereby complicating the interpretation of data generated from experiments conducted by using such a scaffold (Johnson et al., 2007). To overcome this issue, denuded ECM scaffolds from various tissues have been generated (Macchiarini et al., 2008). These scaffolds, when combined with colonies of seeded stem cells, can reconstitute normal tissues with reasonable fidelity (Lutolf et al., 2009). ECMs have also been isolated and extracted from various tissues, such as small intestine, skin (from cadavers), pancreas and breast (Rosso et al., 2005), and these ECMs have been used to engineer skin grafts (Badylak, 2007), enhance wound healing and to study tumour progression. Despite the enormous efforts, however, commercially available matrices still do not fully mimic the organizational features of native ECM.

6.1.6 Matrix production from ECM proteins synthesized by human fibroblasts

The goal is to obtain culture dishes entirely coated with a homogenous, naturally produced, BM-like matrix by allowing fibroblasts to produce and deposit their own ECM while avoiding any extraction or enzymatic

procedures that may alter or denature the "natural" native ECM constitution and molecular organization. Published methods have described the generation of 3D matrices from corneal endothelial and endodermal cells (Vlodavsky, 2001), and a similar approach was later applied to both established and primary fibroblast cell lines (Beacham et al., 2007, Klimanskaya et al., 2005). Fibroblasts require culture for several days at high cell density to generate 3D matrices and evolve "3D-matrix adhesions", namely focal and fibrillar adhesions. The former enable firm linkage to relatively rigid substrates (Burridge and Chrzanowska-Wodnicka, 1996) and co-operate with the latter to generate fibrils from pliable fibronectin (Katz et al., 2000). The resulting matrices closely resemble *in vivo* mesenchymal matrices and are composed mainly of fibronectin fibrillar lattices. Arguably, such matrices enable more physiologically relevant cell-matrix interactions and signalling, and are therefore potentially superior to conventional 2D substrates (Cukierman et al., 2001).

The phenotype of cultured fibroblasts is extremely important for the successful preparation of 3D matrix-coated dishes and should be closely monitored by cell morphology. The fibroblasts should be well-spread and flat under sparse culture conditions. Therefore, the cultures should not be allowed to overgrow to avoid the detachment of fibroblasts.

6.1.7 HDF-produced matrix as a substrate for epidermal differentiation of hESCs

In this chapter, it was hypothesized that ECM produced by normal HDFs could serve as an efficient inducer of epidermal differentiation by mimicking the natural keratinocyte environment, i.e. BM of human skin, in combination with appropriate differentiation conditions and induction medium.

An additional consideration is that for hESCs culture ECM produced by HDFs has a distinct advantage over currently commercially available matrices, such as Matrigel (BD). Matrigel is an ECM proteins mixture secreted by EHS mouse sarcoma cell and, therefore, the culture of hESCs on this substrate cannot be considered fully xeno-free. Therefore, in order to further improve culture conditions of the KCL-002 hESC line, undifferentiated hESC colonies had to be adapted to grow on HDF-ECM.

6.2 METHODS

6.2.1 HDF culture

HDFs were cultured in high-glucose DMEM (Invitrogen) with 10% FBS. Culture stocks were maintained at 80% confluence, and subcultured at a 1:20 dilution approximately once a week. The medium was aspirated and the flasks were rinsed with Ca^{2+} and Mg^{2+}-free PBS (Invitrogen). TrypLE™ Express (Invitrogen) was added to the flasks and incubated for 5min at 37°C. After that, culture medium was added and the cell aggregates were

broken down by gentle pipetting. The cell suspension was then transfered into a 15ml conical tube and centrifuged at 400g for 5min. The medium was aspirated and the pellet resuspended in the appropriate volume of culture medium according to the desired split ratio. Cells were grown in a 37°C incubator at 5% CO_2 until confluent.

6.2.2 Plating HDFs for matrix deposition

The cells were collected from a semi-confluent flask by trypsinization and resuspended in 10ml of matrix medium. The cells were counted using a haematocytometer, and the HDFs were seeded in 24 well plates at a density of 10^5 cells per 200mm² surface area. After 24 hours, the medium was replaced with fresh matrix medium containing 500µg/ml of L-ascorbic acid (tissue culture tested; Sigma). This addition was made because a high concentration of ascorbic acid after the cells have been plated can potentially increase the matrix thickness in primary cell cultures (Beacham et al., 2007). Ascorbic acid should be freshly prepared just prior to use as a 1000× stock concentration of 50mg/ml in PBS and then further diluted with medium to yield a final working concentration, and then filter-sterilized prior to use. After 48hrs, the medium was replaced with fresh matrix medium containing 50µg/ml of ascorbic acid. Ascorbic acid degrades over time in culture, so a media change was performed every 48hrs for a further 7 days. At this point matrices, should be at least 10µm thick. At this thickness, three-dimensionality should be achieved, and thus they are ready to be extracted.

6.2.3 Extraction of HDF-derived 3D matrices

The medium was carefully aspirated and the plates were washed gently with PBS by touching the pipette against the dish wall. Pre-warmed extraction buffer was then added and the plates were placed at 37°C for 3-5 min until no intact cells were visualized. The extraction buffer was composed of 0.5% (v/v) Triton X-100 and 20mM NH4OH (Sigma) in PBS. Cell lysis can be observed using an inverted microscope. PBS was slowly added to dilute the cellular debris. This should be carried out gently to prevent turbulence that can dislodge the freshly denuded matrix-layer from the surface. The diluted cellular debris was then cautiously aspirated making sure that the matrix remained hydrated at all times. The wash step was repeated twice. The matrix coated plates can be used immediately or covered with PBS containing antibiotics, sealed with Parafilm and stored for up to 2 weeks at 4°C.

6.2.4 Immunofluorescence analysis to assess the protein expression in HDF-produced ECM

To test for protein expression in the native de-cellularized matrices produced by HDFs, the plates were fixed with 4% PFA in PBS for 20min at room temperature. ECM is a scaffold mesh of proteins, therefore no permeabilization or blocking was necessary. The plates were then washed with PBS twice and incubated with primary antibodies for 1 hour at room temperature. At the end of this incubation two washes with PBS were performed and the plates incubated with secondary antibodies for a further

30min. Both primary and secondary antibodies dilutions were done in Ca^{2+} and Mg^{2+} PBS. Primary antibodies used in this chapter are outlined in Table 16. One drop of ProLong Gold antifade reagent with DAPI (Invitrogen) was added to simultaneously mount and confirm the absence of nuclei in the HDF-ECM produced. To allow for visualization of immunofluorescent staining, glass coverslips (13 mm diameter; Scientific Laboratory Services) were put on top of the cells in the stained wells. The samples were visualised with a fluorescence microscope. Images were taken with a CCD camera, and processed with Photoshop CS5 (Adobe). The immunofluorescent analysis was carried out each time HDF-derived matrix was produced for quality control.

Protein group	Antibody	Supplier	Host	Isotype	Conc.	Antigen localization
Epidermal markers	Anti-Keratin 14	Covance	Rabbit	IgG	1µg/ml	Intracellular
	Anti-Keratin 5	Covance	Rabbit	IgG	1µg/ml	Intracellular
	Anti-Keratin 18	Millipore	Mouse	IgG1	20µg/mL	Intracellular
	Anti-Keratin 19	Millipore	Mouse	IgG1	5µg/ml	Intracellular
	Anti-Involucrin (SY5)	Sigma	Mouse	IgG1	1:100	Intracellular
	Anti-Integrin α6	Abcam	Mouse	IgG2b	1µg/ml	Transmembrane
	Anti-p63	Abcam	Rabbit	IgG	5µg/ml	Intranuclear
	Anti-Desmocollin2 (H-50)	Santa Cruz	Rabbit	IgG	4µg/ml	Extracellular
Pluripotency markers	Anti-Oct3/4 (C-10)	Santa Cruz	Mouse	IgG2b	2µg/ml	Intranuclear
	Anti-Nanog	Abcam	Rabbit	IgG	1µg/ml	Intranuclear
	Anti-Tra 1-60	Millipore	Mouse	IgM	8µg/ml	Extracellular
	Anti-SSEA-4	DSHB	Mouse	IgG3	1:50 stock	Extracellular
ECM markers	Anti-Fibronectin	Sigma	Rabbit	IgG	1:100	ECM protein
	Anti-Laminin-332	Sigma	Rabbit	IgG	1:50	ECM protein
	Anti-Collagen IV	Sigma	Mouse	IgG1	1:500	ECM protein
	Anti-Collagen VII	Sigma	Mouse	IgG1	1:1000	ECM protein
	Anti-Collagen I	Abcam	Rabbit	IgG	1:80	ECM protein

Table 16 Primary antibodies used Chapter 6.
The penultimate column indicates primary antibody working concentration (conc.)

6.2.5 Adaptation of the KCL-002 hESC line for growth on HDF-ECM as a substrate

Undifferentiated colonies grown on Matrigel (BD) were enzymatically passaged using dispase, as described previously, and transferred onto native de-cellularized HDF-ECM. The cells were grown in mTeSR1 (Stem Cell Technologies) and the medium was changed every 2 days. Undifferentiated hESCs were grown for up to 3 passages before assessing pluripotency marker expression by immunocytochemistry.

6.2.6 Immunocytochemistry for pluripotency marker proteins

For each marker combination duplicate wells were fixed and stained (technical replicates). The immunocytochemistry protocol used for this purpose is outlined in detail in Chapter 2 (Section 2.2.7). Primary antibodies used in this chapter are described in Table 16.

6.2.7 Alkaline phosphatase staining

Endogenous alkaline phosphatase activity in hESC cultures was tested using the ELF detection assay (ATCC Stem Cell Centre, Teddington, UK), according to the manufacturer's instructions. Cleavage of the phosphatase substrate generates a very fine precipitate at the site of alkaline phosphatase activity. In this assay, the weak blue fluorescence of the substrate is converted to intensely fluorescent yellow-green precipitate at

the site of enzymatic activity. The excitation/emission of the reaction product is 345/530nm and the staining appears yellow-green against a blue background when visualized with a Hoechst/DAPI filter.

hESCs grown on HDF-ECM were fixed with 4% PFA for 10min at room temperature and then rinsed in PBS. Permeabilization was done in 0.2% Tween-20 in PBS for 10min at room temperature and rinsed in PBS for 10min. Following the washes, 50µl of the filtered diluted Phosphatase Substrate was added to the cells while at the microscope to monitor the progress of the reaction. The reaction was usually completed within 30 to 90s and rarely required longer than 5min. The reaction is stopped by submerging the sample in wash buffer (PBS with 25mM EDTA and 5mM levamisole, pH 8.0) three times over 15min with gentle agitation. The samples were then mounted with the mounting medium supplied with the kit. Staining was visualized through a standard Hoechst/DAPI long pass filter set, which provides the appropriate UV excitation and transmits wavelengths greater than 400nm. With this filter set, the yellow-green signal appears very distinct against a blue background.

6.2.8 HDF-ECM differentiation protocol

The outline of the differentiation protocol is schematically represented in Figure 42. Undifferentiated colonies were dissociated with 1 mg/ml dispase for 5min at 37˚C, as described previously (section 2.2.5), and cells were seeded onto freshly made HDF-ECM at a density of 5-10x10^3 cells per cm^2. The cells were grown in mTeSR1 for 4 days before switching to

differentiation induction medium (day 11 of differentiation protocol). Induction medium was composed of DMEM:Ham's F-12 medium (at a 1:1 ratio) (Invitrogen) supplemented with 1µM ATRA (Sigma) and 25ng/ml BMP-4 (R&D). ATRA dilutions were performed in DMSO to a stock concentration of 10mM. The medium was changed daily for 7 days before switching to DKSFM (Invitrogen). After a further 7 days (day 25 of differentiation protocol) the culture medium was aspirated and differentiated colonies were dissociated with 1mg/ml dispase for 3-5min at 37°C. The dispase was then aspirated and replaced with DKSFM (Invitrogen). The epithelial-like sheets were scraped off with a cell scraper and centrifuged at 200g for 5min before being resuspended in DKSFM. The cells were replated on dishes pre-coated with type IV collagen and a rapid-adhesion enrichment method was applied as described previously (section 4.2.5). The resulting rapidly-adhering cell population was cultured for a further week in DKSFM before final analysis.

The differentiation protocol was independently repeated 3 times with hESCs at different passage number (n=3 biological replicates).

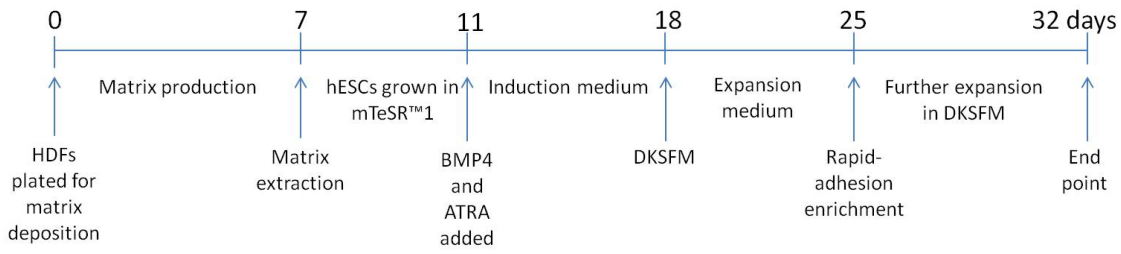

Figure 42 Schematic representation of the differentiation protocol using HDF-ECM as substrate

6.2.9 Subculture, cryopreservation and recovery of pKPCs

The pKPCs obtained at day 32 of the differentiation protocol were further expanded in DKSFM. At this stage cells were subcultured enyzymatically, by incubating with TrypLE Express (Invitrogen) at 37°C for 4min. Following centrifugation at 300g for 5min, the cells were counted using a haemocytometer. The cells were then either seeded at a density of 10,000 cells/cm^2 on gelatin-coated tissue culture plates in DKSFM for further expansion, or cryopreserved by re-suspending 10^6 cells in 1ml of DKSFM 10% FBS and 10% DMSO. The suspenson was aliquoted into labeled cryovials and stored at -80°C overnight before being transferred into a liquid nitrogen vapor tank. To recover pKPCs from liquid nitrogen, cryovials were quickly thawed in a 37°C waterbath until only a small frozen pellet remained. The cryovial was wiped with 70% ethanol to sterilize prior to opening. The contents were transferred into a 15ml conical tube and 5ml of pre-warmed DKSFM was added dropwise to the tube, gently mixing as the medium is added. The cells were centrifuged at 300g for 5min. The medium was aspirated and the pellet resuspended in 5ml of DKSFM. Following the cell count, 10,000 cells/cm^2 were plated on gelatin-coated tissue culture plates in DKSFM.

6.2.10 Immunocytochemstry for epidermal differentiation markers

Immunocytochemistry was carried out at days 7, 14, 21 and 25 of the differentiation protocol, as described previously in Section 2.2.7. At each

time point 3 wells of a 24 well plate were randomly chosen for immunocytochemical analysis (3 technical replicates). Primary antibodies used in this chapter are outlined in Table 16.

6.2.11 Quantification of immunofluorescence data and statistical analysis

Quantification of immunofluorescence data was performed as described previously in section 5.2.9. Two-way Analysis of Variance (ANOVA) statistical test was used to compare 3 groups of parametric, unpaired data sets. Defining statistically significant differences between groups was achieved by applying the Bonferroni post-hoc tests to data analyzed by ANOVA.

6.2.12 Direct flow cytometry for the α6-integrin subunit and keratin 14

The cells obtained at day 32 of the differentiation protocol were harvested by trypsinization using TrypLE Express (Invitrogen), and centrifuged at 300g for 5min. The cell suspension was adjusted to a concentration of 0.5-1 $x10^6$ cells/ml in ice-cold FC buffer (1% BSA/PBS) and 100µl of cell suspension was added to each sample tube. Polystyrene round-bottom 12x75mm Falcon tubes (BD) were used for cell staining. A minimum of 1 $x10^6$ cells/per sample was analyzed. Surface antigen staining was performed first: as recommended by the supplier, a volume of 20µl of PE-conjugated rat

IgG2a anti-CD49f (α6-integrin) antibody (BD Pharmingen, 555736) was added per sample. The tubes were vortexed and incubated for 30min at room temperature in the dark. The samples were then washed in 2ml FC buffer at 300g for 5min. Prior to intracellular staining, the samples were fixed and permeabilized. To fix the cells, 100µl of 4% (v/v) PFA in PBS was added to each sample tube and kept at 4°C for 10min. The cells were then washed with 2ml FC buffer at 500g for 5min. The supernatant was then poured off leaving approximately 100µl of buffer containing the cell pellet. Permeabilization was done with 500µl ice-cold 0.1% Triton-X 100/PBS on ice for 20min. The samples were then washed with 2ml FC buffer at 500g for 5min. A recommended volume of 10µl/per sample of FITC-conjugated mouse IgG3 anti-Keratin 14 antibody (Abcam, ab77684) was added. The tubes were vortexed and incubated for 30min at room temperature in the dark and then washed with 2ml FC buffer at 500g for 5min. The supernatant was poured off and the cell pellet was resuspended in 500µl acquisition buffer composed of 1% BSA and 0.1% (w/v) sodium azide in PBS. The cells were kept on ice until analysis and data was acquired using a BD Canto flow cytometer within 12 hours.

Isotype controls were used to confirm that the primary antibody binding was specific and not a result of non-specific Fc receptor binding or other protein interactions. The following isotype controls were used: mouse IgG3 FITC (BD Pharmigen, 556658) for anti-keratin 14 antibody and rat IgG2a PE (BD Pharmigen, 559317) for anti-CD49F antibody. Both isotype control antibodies were used at the same concentration (1µg/1 x10^6 cells) and under the same conditions as the test stain.

The BD™ CompBeads Sets Anti-Mouse Ig (552843) and Anti-Rat Ig (552844) were used as compensation controls. Compensation beads are polystyrene microparticles, which are used to optimize fluorescence compensation settings for multicolour flow cytometric analyses. The set provides two populations of microparticles: the anti-mouse/rat Ig κ particles that bind any mouse/rat κ light chain-bearing immunoglobulin, and the negative control (FBS), which has no binding capacity. When mixed together with a fluorochrome-conjugated mouse or rat antibody, the beads provide distinct positive and negative (background fluorescence) stained populations, which can be used to set compensation levels and more accurately establish corrections for spectral overlap for double-staining analysis.

The beads were used as follows: 1 full drop (approximately 60µl) of the BD™ CompBeads Negative Control (FBS) and 1 drop of the BD™ CompBeads anti-mouse or anti-rat Ig beads were added to a tube containg 100µl of FC buffer. An optimal amount of each primary antibody to be tested was then added into an appropriate tube and incubated for 20mins at room temperature in the dark. Next 2ml FC buffer was added to each tube followed by centrifugation at 200g for 10min. The supernatant was discarded and the bead pellet resuspended in 500µl acquisition buffer. Gating was done on the single bead population based on FSC (forward-light scatter) and SSC (side-light scatter) characteristics. Flow rate was adjusted to 200 events per second. For each given fluorochrome-conjugated antibody dot plots were done as appropriate, i.e. an FL1 vs. FL2 dot plot was used to set compensation for FITC-conjugated anti-keratin 14 antibody. A quadrant

gate was placed such that the negative bead population was in the lower left quadrant and the positive bead population was in the upper or lower right quadrant, and the compensation values were adjusted until the median fluorescence intensity of each population was approximately equal.

The BD™ CompBeads Negative Control (FBS) was used as a negative control to set up the flow cytometer voltage.

6.2.13 Colony forming efficiency (CFE) assay

The pKPCs obtained at day 25 of the differentiation protocol were harvested and plated at a density of 5000 cells/cm² in 3 wells of a 6-well plate coated with gelatin. The number of viable cells at the start of the assay was determined with Trypan Blue and counted in a haematocytometer chamber. The cells were grown in DKSFM with media changes performed every 48 hours. After 7 days in culture, cells were stained with 0.25% crystal violet (Sigma). Colonies, defined as ≥50 cells, were counted. The colony-forming efficiency was calculated according to the following formula (Boyce and Ham, 1983): CFE=Number of colonies x 100/Number of live cells plated. CFE was calculated separately for each of 3 wells and the mean was calculated. The assay was performed in triplicate (technical replicates) on pKPCs obtained from 3 independent rounds of differentiation protocols (n=3 biological replicates) and the results were compared to CFE of NHKs (positive control) using Students' t-tests.

6.2.14 MTT Proliferation assay

Proliferation efficiency was calculated using a MTT assay. The MTT assay utilizes a colometric reaction secondary to succinate dehydrogenase activity in mitochondria and is a sensitive, reliable indirect measure of cell viability and proliferation (Mosmann 1983). Intracellular dehydrogenases of actively proliferating cells convert the yellow tetrazolium MTT to purple formazan. The optical density (O.D.) of the resulting formazan product can be easily measured and quantified.

The differentiated cells at day 25 were seeded at a density of 5000 cells/ml in 96-well plates in 200µl DKSFM medium. After 48 hours 10µl of 5mg/ml MTT (Sigma) was added to each well and incubated at 37°C for 2.5 hours. The crystal formazan product was dissolved by adding 200µl DMSO (Sigma) and a further incubation for 15min. O.D. was measured at 570nm with an ELISA plate reader, using 620nm filter as a reference wavelength. The MTT assay was performed in triplicate (technical replicates) on pKPCs obtained in 3 independent rounds of the differentiation protocol (n=3 biological replicates). Negative controls were performed with empty wells. The results were then compared using Student's t-tests to those obtained with NHKs (positive controls).

6.3 RESULTS

6.3.1. HDF-produced 3D ECM *in vitro* is similar in composition to BM *in vivo*

To assess the quality and composition of native ECM produced by HDFs, and also to confirm the complete removal of cells from the resulting matrices, immunofluorescent analysis for skin BM-specific ECM proteins was carried out (Figure 43). Positive immunofluorescent staining revealed the presence of a dense meshwork of fibronectin fibres, numerous fine types IV and VII collagen fibrils, as well as type I collagen and laminin-332. DAPI was used to confirm the absence of HDF nuclei. Figure 44 shows the relevant positive and negative controls for characterization of HDF-produced ECM by immunofluorescence. The next step was to adapt the KCL-002 hESC line to grow on these matrices as a substrate.

6.3.2 KCL-002 hESC line maintains pluripotency on HDF-ECM

To assess the pluripotency of KCL-002 hESC colonies grown on HDF-ECM, the cell morphology and expression of pluripotency marker proteins were examined. When cultured on HDF-ECM in the presence of mTeSR™1, undifferentiated KCL-002 colonies retained their typical undifferentiated morphology, notably displaying well-defined colony edges, a tightly packed colony and the absence of differentiated cells. The colonies tested positive for presence of pluripotency markers Oct-3/4, Tra 1-60, SSEA-4, Nanog and Alkaline Phosphotase (Figure 45). These results confirmed that KCL-002

hESCs could be cultured on HDF-ECM, providing a rationale for testing the efficiency of epidermal differentiation using this substrate.

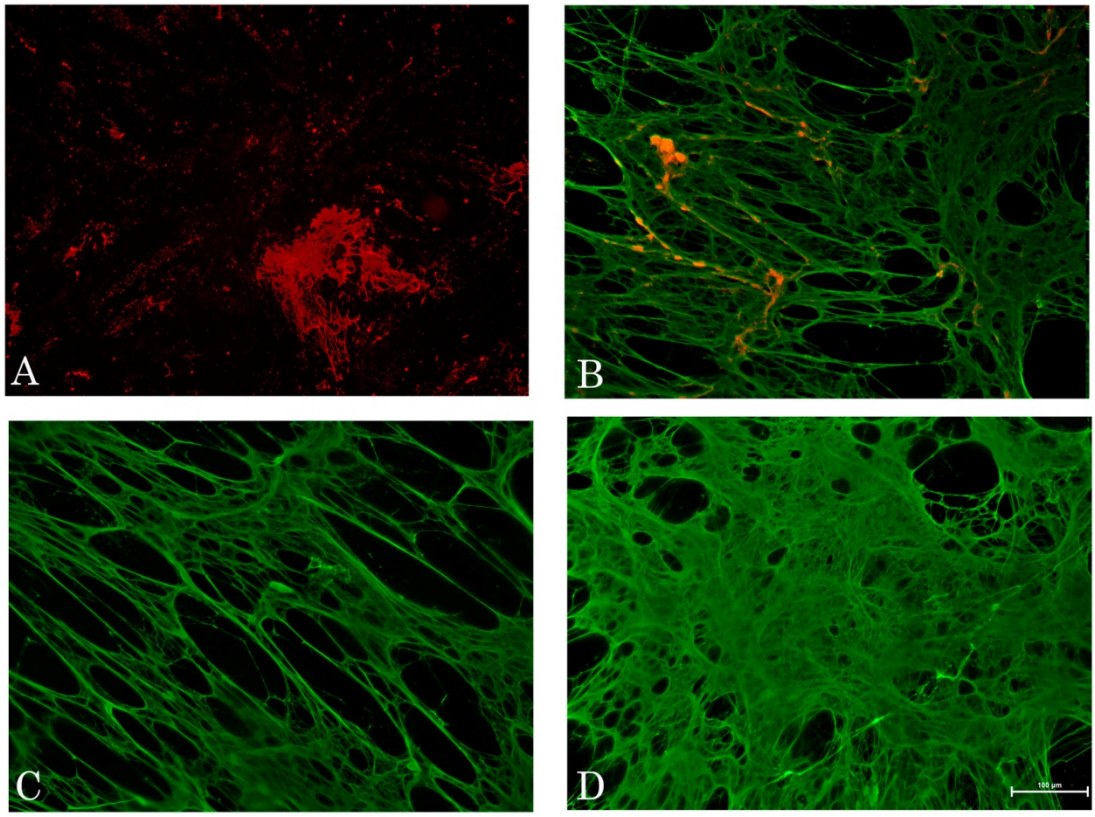

Figure 43 Characterization of HDF-produced ECM by immunofluorescence. **A:** Laminin-332 (red); **B:** Collagen type IV (green) and Collagen type I (red); **C:** Collagen type VII (green); **D:** Fibronectin (green). No HDF nuclei are observed in extracted matrices, as determined by the absence of DAPI staining. Scale bar=100µm

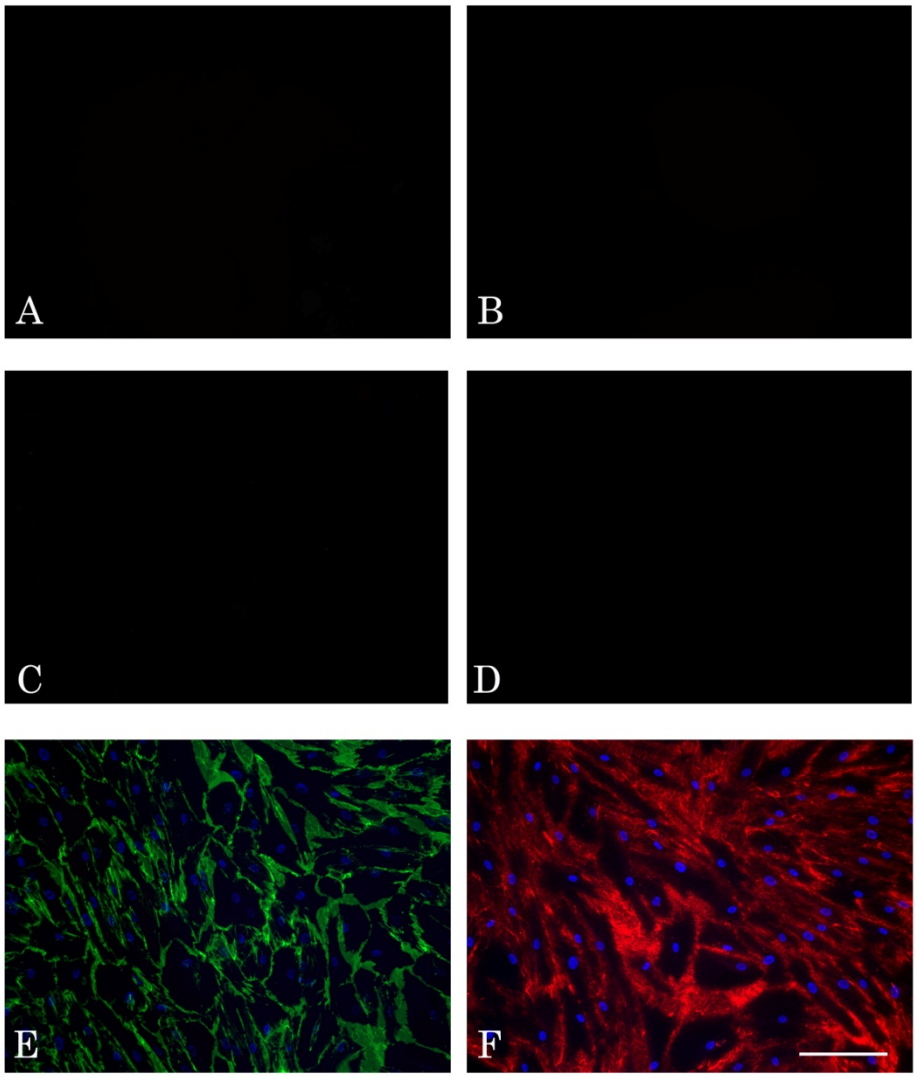

Figure 44 Relevant controls for immunofluorescent analysis of HDF-produced ECM
A: No anti-laminin 332 primary antibody negative control; **B:** No anti-collagen type IV primary antiody negative control; **C:** No anti-collagen type VII primary antibody negative control; D: No anti-fibronectin primary antibody negative control; **E:** Collagen type IV expression by HDFs prior to matrix extraction ; **F:** Laminin-332 expression by HDFs prior to matrix extraction. Nuclei of HDFs stain blue by DAPI. Scale bar=100μm.

6.3.3 Morphology and expression of epidermal markers during the course of differentiation protocol

Epidermal differentiation, utilizing HDF-ECM as a natural substrate and BMP-4 together with ATRA, was initially evaluated by morphological observations and immunocytochemistry. After 7 days of differentiation

induction (Figure 46), the peripheral cells of each colony spread out greatly and became flattened, whereas more centrally located cells remained more tightly packed and appeared to be fast dividing, continuing to grow exponentially until the cultures became confluent. These colonies stained positively for extracellular desmosomal marker DSC 2 and cytoplasmic keratins 19, 5 and 14. Interestingly, the expression of keratins 5 and 14 was more intense in the peripheral cells. By day 25, large sheets of cells with more typical epithelial morphology could be observed (Figure 47). A few very large and flat cells could be seen at the edges of these putative epithelial sheets. Those cells were either only very dimly positive or negative for both keratin 14 and p63 expression. On the contrary, the central parts of the sheets displayed bright p63 positivity (Figure 47B). When the putative epithelial sheets were scrapped off and the cells with a higher replicative potential were selected by rapid adhesion to type IV collagen, some rapidly adherent cells formed compact colonies brightly positive for p63 transcription factor expression, as well as keratin 14 (Figure 48). These colonies grew into large epithelial-like sheets and retained their morphology and keratin 14 and p63 markers expression (Figure 49). Further subcultures could be done on gelatin without loss of marker expression for another 5 passages. Furthermore, pKPCs could be cryopreverved and recovered without the loss of marker expression (Figure 50). These initials results suggested that the method was efficient in inducing epidermal differentiation from hESCs although further confirmation would be necessary.

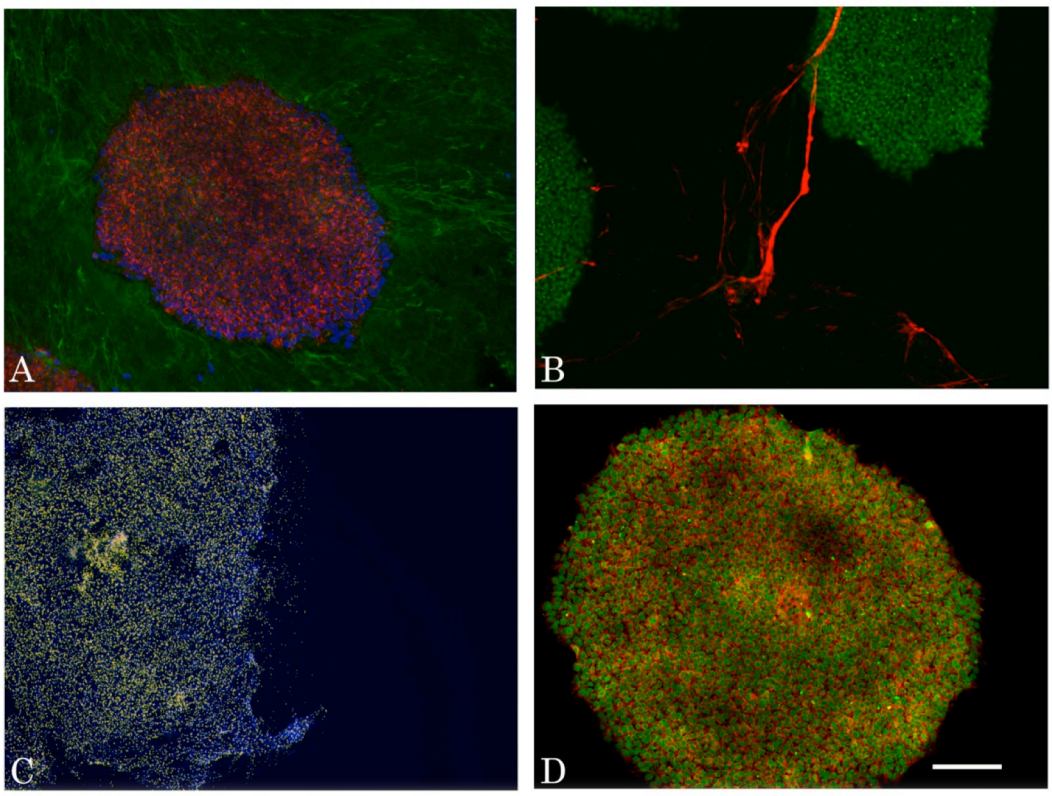

Figure 45 Expression of pluripotency-associated markers in KCL-002 hESC colonies grown on HDF-ECM.
A: Tra 1-60 (red) positive colony can be seen on fibronectin (green). DAPI nuclear staining (blue); **B:** Nuclear Nanog staining (green) and patches of laminin-332 (red); **C:** Alkaline phosphatase staining; **D:** Nuclear Oct-3/4 staining (green) and extracellular SSEA-4 (red; Scale bar=100µm.

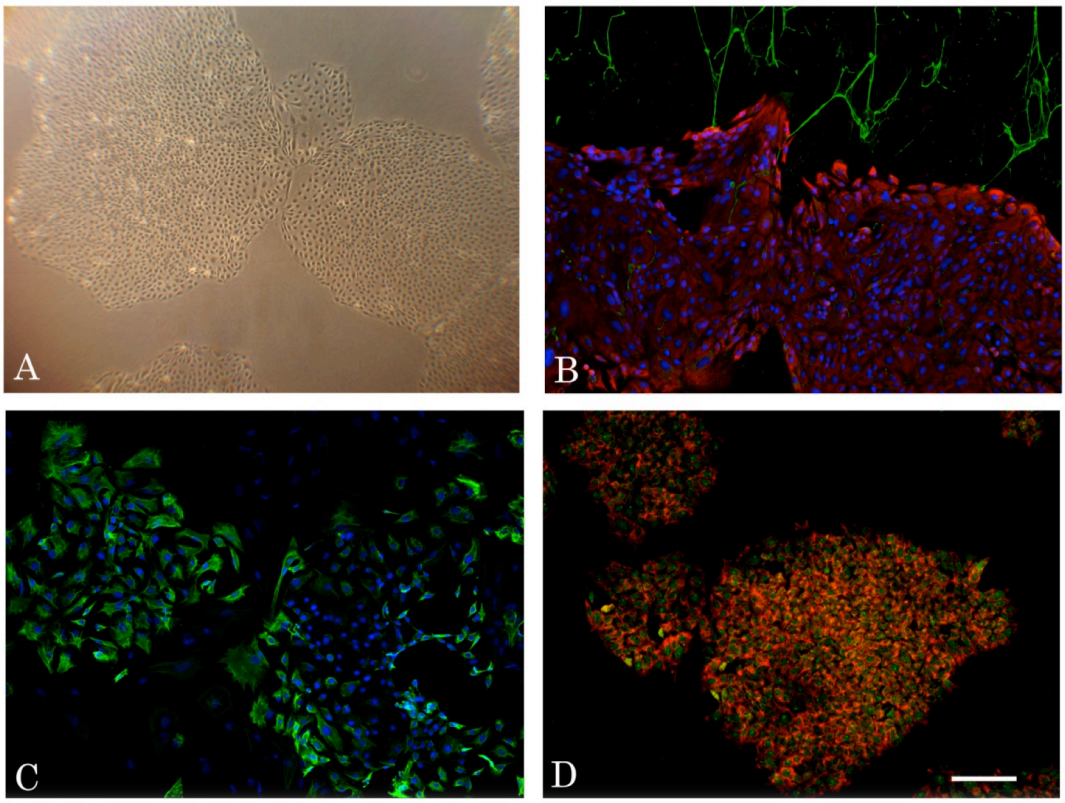

Figure 46 Morphology and expression of epidermal markers after 7 days of induction (day 18 of differentiation).
A: Morphology of a colony at day 18. The peripheral cells appear spread out and flattened, whereas centrally located cells remain tightly packed; **B:** Keratin 14-positive colonies (red) on type IV collagen (green); **C:** Keratin 5-positive cells (green) begin to appear at the periphery; **D:** Extracellular DSC 2 staining (red) and cytoplasmic keratin 19 (green). Nuclei are visualized with DAPI (blue). Scale bar=100µm

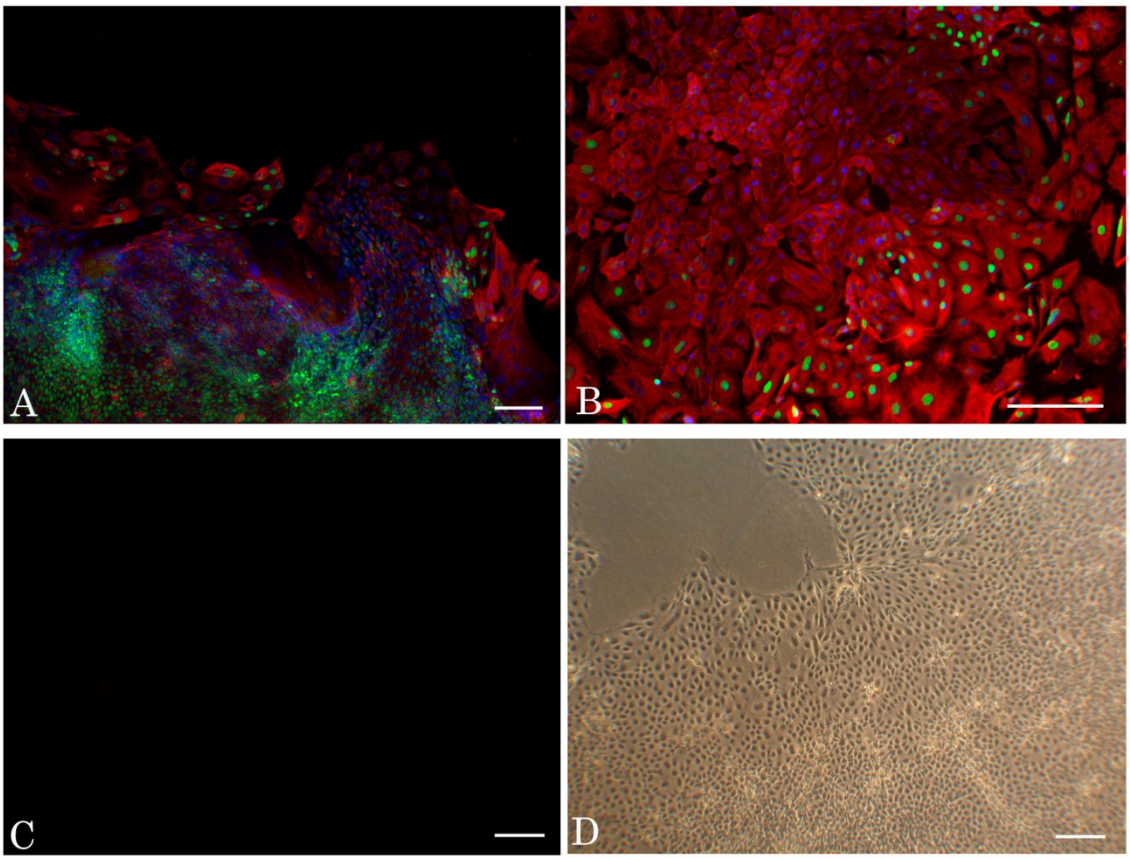

Figure 47 Expression of epidermal markers and morphology of differentiating cells at day 25 before enrichment.
A: Bright nuclear p63 staining (green) can be seen in the central parts of the putative epithelial-like sheets. The vast majority of cells are keratin 14-positive (red). Note that the large cells at the periphery are only dimly positive for both markers. DAPI nuclear staining (blue); B: High magnification of central p63-positive cells. C: No primary antibody negative control. D: The sheets display a typical epithelial morphology. Scale bar=100µm.

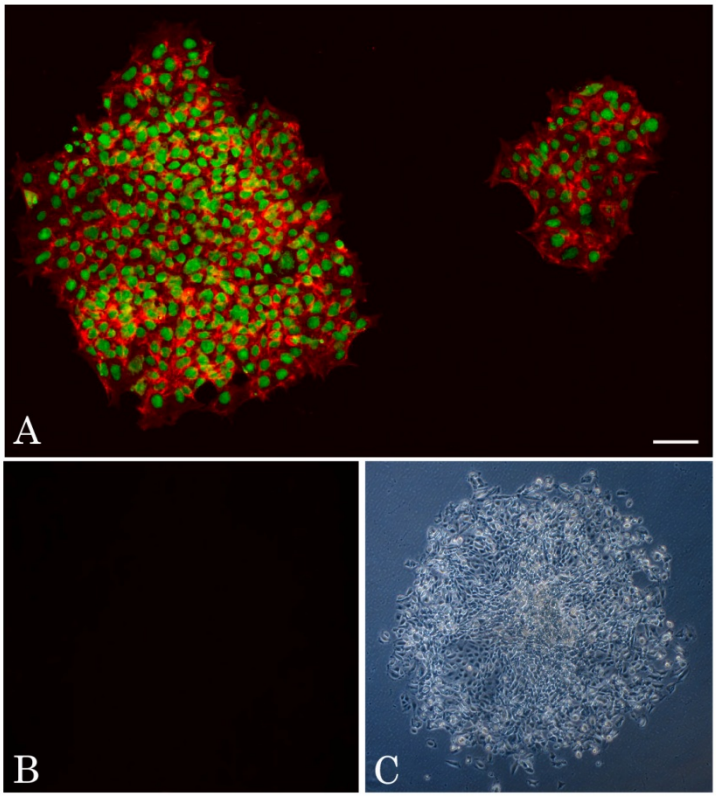

Figure 48 Colony of enriched pKPC population at day 27.
A: The colony displays an intense positivity for nuclear p63 (green) and cytoplasmic keratin 14 (red); B: No primary antibody negative control; C: Morphological appearance of the colony. Scale bar=50μm.

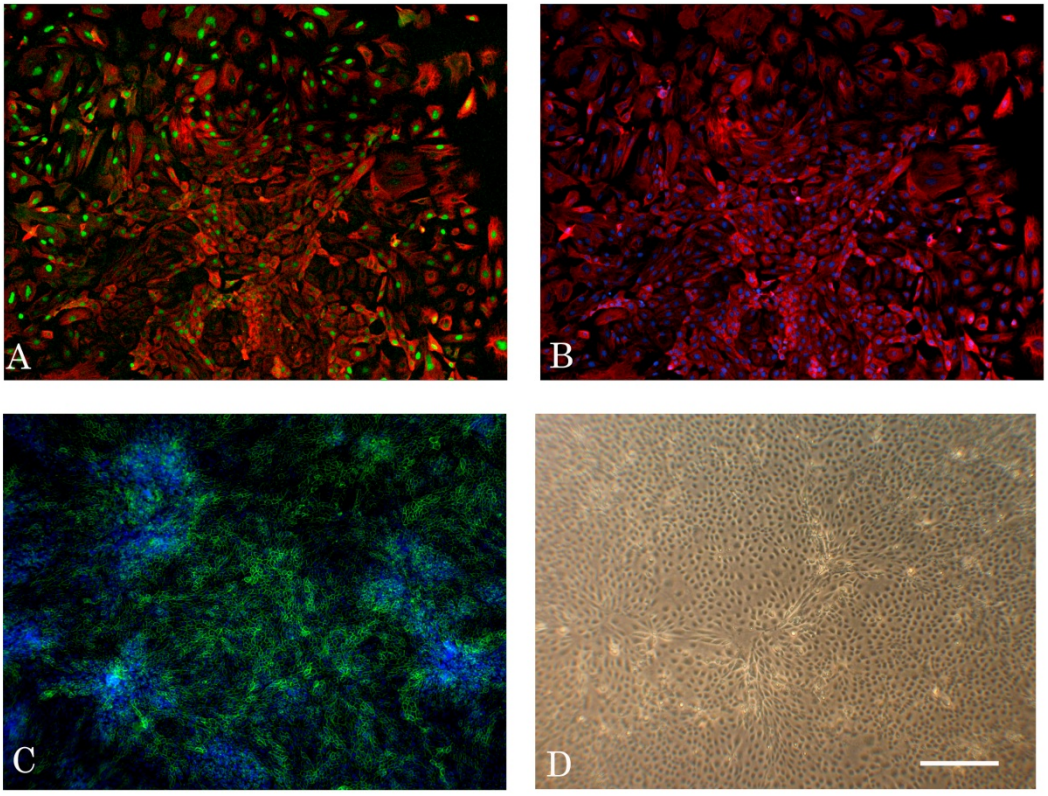

Figure 49 Characterization of pKPCs at the end point (day 32) of differentiation.
A: Many brightly p63- (green) and keratin 14-positive (red) cells can be seen. B: DAPI staining (blue) confirms the nuclear co-localization of p63. C: Keratin 5 staining (green). D: Morphology of epithelial-like sheets. Scale bar=100µm.

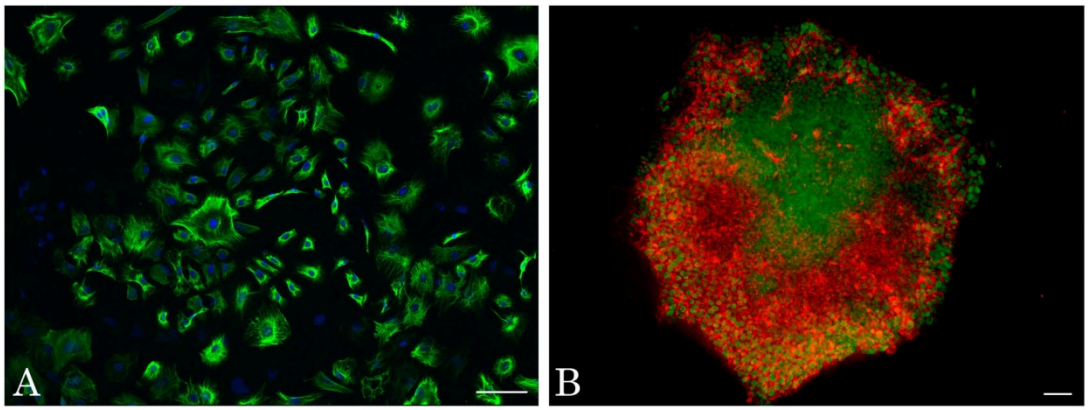

Figure 50 Characterisation of recovered pKPCs showing sustained marker expression.
A: Keratin 5 positivity; **B:** Tightly packed colonies showing keratin 14 (red) and p63 (green) expression. Scale bar=50µm.

6.3.4 HDF-ECM protocol supports hESC differentiation into epidermal precursors

In order to evaluate the efficiency of the HDF-ECM protocol, immunofluorescent data was quantified and compared to results obtained during PA6 stromal cell-induced differentiation (described in Chapter 3). The cells positive for keratin 14 were counted against the total number of DAPI-positive cells, and the following results were obtained (Figure 51). At day 18, 85.1±1.6% of cells were positive for keratin 14 protein expression. By day 25 that number increased to 91.7±2.3%. Statistical analysis confirmed a significant difference between the efficiency of production of keratin 14 positive cells at both time points. Two-way ANOVA test and the Bonferroni post-hoc test revealed that the HDF-ECM protocol was significantly more efficient in producing keratin 14 positive cells compared to both PA6 CM and indirect co-culture differentiation protocols. The statistical difference after 7 days of induction gave $p<0.0001$ and $p<0.001$ in comparison with PA6 CM and Transwells protocols, respectively. After 14 days of induction, the p values were $p<0.0001$ and $p<0.05$ when compared to PA6 CM and Transwells protocols, respectively.

The high efficiency of differentiation on HDF-produced ECM in the presence of BMP-4 and ATRA was further validated with flow cytometry and CFE functional analysis.

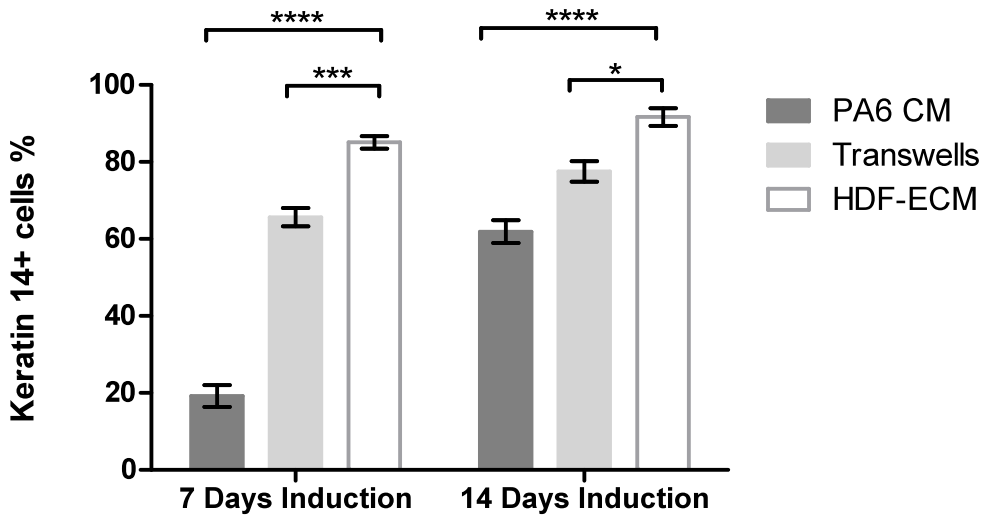

Figure 51 Comparison of epidermal differentiation induced by HDF-ECM vs. secreted PA6 growth factors by quantification of immunofluorescent data.
Percentage of keratin 14-positive cells as calculated per 3 independent fields of 100 DAPI-positive. n=3. Quantification was done at two different time points, 7 and 14 days post-induction. Values expressed as mean ± SEM. Significant difference in keratin 14 protein expression was observed when the HDF-ECM differentiation method was compared to the two methods described in Chapter 3. **** $p<0.0001$, *** $p<0.001$, * $p<0.05$

6.3.5 Expression of epidermal markers is sustained in expanded pKPCs

To quantify the presence of epidermal markers α6-integrin and keratin 14, flow cytometry analysis was carried out (Figure 52). At passage 1 (day 32 of differentiation protocol), all of the pKPC cells were positive for keratin 14 with 80.2% also positive for the α6-integrin subunit. When flow cytometry was repeated at passage 5, similar results were observed with all cells remaining positive for keratin 14 and 77.7% also expressing α6-integrin. These results indicate that a homogenous population of keratin 14-positive pKPCs is obtained at the end of the HDF-ECM induced epidermal differentiation protocol. Furthermore, this population retains epidermal

marker expression following passaging and expansion, thus indicating a high proliferation potential of the pKPCs obtained. In order to further assess the proliferation potential of the resultant pKPC population, functional clonogenic and MTT proliferation assays were conducted.

6.3.6 CFE of pKPCs is lower than that of NHKs

CFE was measured by the ability of a defined number of pKPCs plated to form viable colonies composed of a minimum of 50 cells. Cell viability was confirmed by staining with crystal blue dye, which binds to DNA. CFE assays were carried out in triplicate (n=3) on pKPCs obtained at day 32 in 3 independent rounds of differentiation and compared to NHKs (Figure 53A). The values are presented as mean±SD. After 7 days of culture pKPCs formed 6.4±1.3 viable colonies vs. 10.6±0.6 in NHK cultures. Unpaired two-tailed Students' t-test returned p=0.0453 indicating statistical significance ($P<0.05$) in the difference between the two values.

6.3.7 pKPCs have a high proliferation potential

Previous differentiation studies reported a low proliferation potential of ESC-derived keratinocytes (Aberdam et al., 2007, Green et al., 2003, Ji et al., 2006, Metallo et al., 2008b), therefore I sought to examine the potential for expansion and proliferation of pKPCs obtained in the HDF-ECM protocol.

The MTT assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate tetrazolium bromide (MTT) into a dark blue insoluble formazan product. The optical density (O.D.) of the resulting product can be measured by ELISA plate reader at 570nm absorbance. MTT assay was carried out in triplicates (n=3) on pKPCs obtained at day 32 in three independent rounds of differentiation and compared to NHKs (Figure 53B). The values are presented as mean±SD. Unpaired two-tailed Students' t-test was used to evaluate statistical significance between the values obtained for pKPCs (0.2±0.03) and NHKs (0.2±0.03). The resulting p=0.1543 indicating no significant statistical difference between the two cell populations.

The collective results described in this set of experiments indicate that when cultured on HDF-ECM, under appropriate differentiation-inducing culture conditions, hESCs can undergo efficient epidermal differentiation.

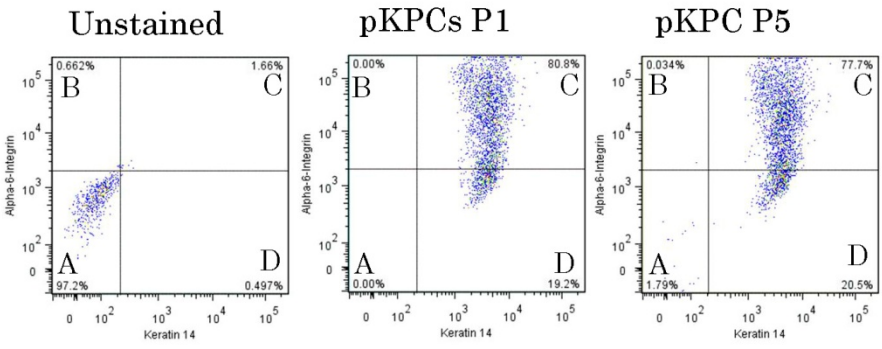

Figure 52 Flow cytometry for keratin 14 and α6-integrin expression in pKPCs at passage 1 (P1) and passage 5 (P5).
A: negative cells; **B:** α6-integrin-positive cells; **C:** Double positive for α6-integrin and keratin 14; **D:** Keratin 14-positive cells.

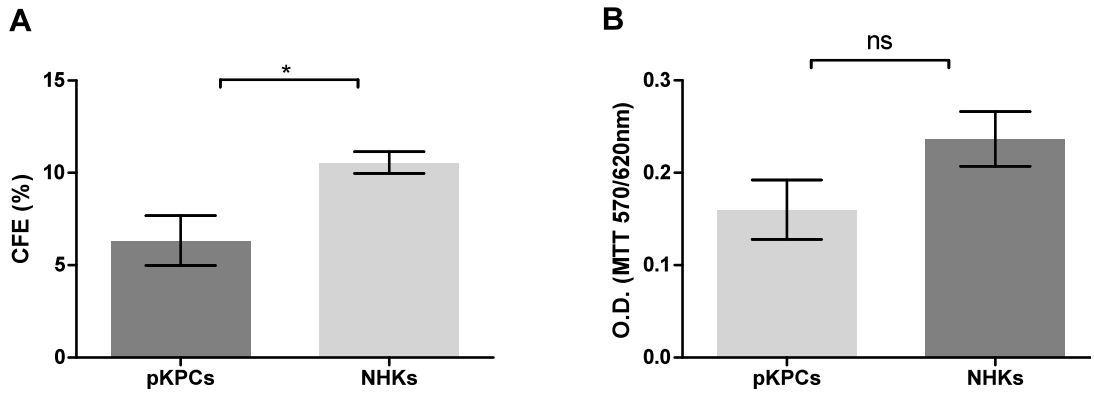

Figure 53 Colony forming efficiency (A) and Proliferation efficiency (B) of pKPCs as compared to NHKs.
Each assay was performed in triplicate on cells obtained from 3 independent repeats of the differentiation protocol (n=3). Values are presented as mean±SD. CFE, Colony forming efficiency. O.D., Optical Density. **p*<0.05, ns-not significant i.e. *p*>0.05.

6.3.8 Technical discussion

A great improvement in the characterization of the HDF-produced ECM would be to perform a protein gel electrophoresis in order to visualize the proteins present. It is important to remember, however, that some proteins known to be major constituents of the ECM are highly glycosylated. For example, *N*-glycosylation of fibronectin is known to protect the protein from proteolytic degradation (Bernard et al., 1982). Therefore, the extracted ECM must be treated with glycosidase to remove these covalent modifications. The enzymatically digested proteins can be extracted from the surface of the culture plate and analysed by SDS-PAGE or two-dimentional electrophoresis. An important consideration is that when dealing with complex samples, such as HDF-ECM, one-dimensional SDS-PAGE does not allow for differential analysis of protein expression, since each band revealed on the gel may contain multiple distinct proteins (Mukhopadhyay et al., 2001). On the other hand, two-dimensional gels only allow for

visualization of proteins of less than 150kDa (Bumke et al., 2003, Lim and Bodnar, 2002), and it is well known that several matrix proteins possess molecular masses above this value and their masses are often further increased by numerous glycosylation events. Therefore, it would be beneficial to identify individual proteins by mass spectrometry, a highly sensitive method that allows for the efficient identification of proteins in complex mixtures (Peng and Gygi, 2001).

Another technical limitation of this study is the fact that the resulting pKPC population was analysed by flow cytometry based on the expression of α6-integrin subunit, a commonly used EpSC marker (Li 1998), which is also highly expressed in undifferentiated hESCs (Xu et al., 2001). It has been shown that over 90% of hESCs grown on Matrigel express α6- and ß1-integrin subunits, while less than 25% of hESCs were found to express the ß4 subunit (Meng et al., 2010). As discussed previously in section 1.8.4, this integrin subunit plays a crucial role in epidermal homeostasis and is abundantly found in human keratinocytes as a part of hemidosmosomes complex (Watt and Hertle, 1994, Margadant et al., 2010). Therefore, flow cytometry analysis for ß4-integrin subunit expression would significantly augment the characterization of pKPCs obtained.

6.4 DISCUSSION

In this set of experiments, I aimed to produce ECM from HDFs that would closely resemble BM of human skin. In addition, I sought to examine the potential of such matrix to support the growth of undifferentiated hESCs.

The main aim of this set experiments was to subsequently evaluate the efficiency of the HDF-ECM produced as a substrate for *in vitro* epidermal differentiation of hESCs when appropriate differentiation-inducing factors (namely BMP-4 and ATRA) were added. Indeed, ECM secreted by normal HDFs appears to resemble human skin BM in expression of several key proteins as evaluated by immunofluorescence analysis. Furthermore, HDF-produced matrix was shown to support the growth and proliferation of the undifferentiated KCL-002 hESC line in the presence of mTESR medium. When hESCs grown on HDF-ECM as a substrate were induced to differentiate by treatment with BMP-4 and ATRA, highly proliferative pKPCs, positive for the basal keratin 14 protein and p63 transcription factor, were obtained. These pKPCs could form large epithelial-like sheets when cultured in keratinocyte medium and sustained basal keratin 14 expression when expanded and passaged. They also displayed a reasonably high clonogenic potential and proliferation capacity, comparable to that in NHKs. These collective results indicate that by mimicking the natural keratinocyte environment, i.e. BM of human skin, ECM produced by normal HDFs can serve as an efficient substrate to direct hESCs towards an epidermal fate.

6.4.1 ECM produced by HDFs is similar in composition to skin BM

Immunofluorescent analysis of the produced HDF matrices revealed the presence of dense meshwork of fibronectin fibres, numerous fine fibrils of

types IV and VII collagen, as well as non-organized batches of type I collagen and laminin-332, confirming the presence of the key ECM proteins of skin BM.

The BM at the DEJ is known to be composed mainly of type IV and VII collagens, several laminins such as laminins-332, -311, and -511, nidogen, and perlecan (Marinkovich et al., 1993, Burgeson et al., 1994). Type IV collagen is the main component of the lamina densa, while anchoring filaments transversing lamina lucida are composed mainly of laminin-332 (Rousselle et al., 1991). Fibronectin is an important constituent of the DEJ (Couchman et al., 1990), which plays an important role in keratinocyte proliferation and differentiation (Adams and Watt, 1989), and type VII collagen forms anchoring fibrils in the sub-lamina densa region (Sakai et al., 1986, Lunstrum et al., 1986). Therefore, basic molecular characterization confirmed HDF-ECM contains the key ECM proteins that are essential for organizing BM of the skin (Inoue, 1989).

However, one significant difference in the composition of HDF-ECM compared to skin BM is the presence of type I collagen in the former. Type I collagen is normally found in the interstitial collagen layers of the dermis (Junqueira et al., 1983, Meigel et al., 1977), and not in the BM. This difference could significantly alter the overall molecular organization of the matrix, and in turn affect the proliferation, migration and differentiation of the dependant cell population. Furthermore, the thickness of HDF-synthesized matrices varies between 8 and 20μm (Beacham et al., 2007). This is considerably more than the thickness of the BM of human skin (0.5-

1.0μm) (Inoue, 1989). This feature also has the potential to alter or affect the biophysical properties of HDF-ECM and, consequently, the vital function of ECM in maintaining the putative EpSC population.

6.4.2 HDF-ECM supports culture and maintenance of pluripotent hESCs

Culturing hESCs on a native de-cellularized matrix produced by fibroblasts of human origin could enable a further optimization of hESCs culture towards xeno-free conditions. As mentioned previously, Matrigel (Kleinman and Martin, 2005, Kleinman et al., 1986) is produced from EHS mouse carcinoma and, despite its effectiveness as a substrate for hESCs, culture cannot be considered xeno-free. Previously, it has been shown that such matrices can indeed support the derivation and propagation of hESCs (Klimanskaya et al., 2005). However, that study utilized MEFs for matrix derivation. The results described in this chapter show that hESCs can be successfully cultured on ECM produced by HDFs while retaining the expression of pluripotency-associated proteins. Very recently, an optimized general protocol for entirely animal-protein free and feeder-free derivation and propagation of hESCs has been developed at King's College London (Ilic et al., 2012). This protocol utilizes native de-cellularized ECM produced by human foreskin fibroblasts and TeSR™2 medium.

6.4.3 HDF-ECM as an inducer of epidermal differentiation of hESCs

The epidermal differentiation protocol utilizing HDF-ECM as a natural substrate and synergetic effects of BMP-4 and ATRA is highly efficient in producing a homogenous population of keratin 14-positive pKPCs, which also express high levels of p63 transcription factor.

Previously, epidermal differentiation was achieved when murine ESCs were seeded on matrix derived from human normal fibroblasts and NIH-3T3 cells (Coraux et al., 2003b). Under these conditions, keratin 14-positive cells were obtained which formed well-defined epidermal layers with appropriate marker expression in an organotypic mouse model. However, the relative percentage of keratin 14-positive cells in that study was only 9.8%, suggesting that differentiation culture conditions could be improved. Recently, refined culture conditions were developed that relied on prolonged time- and concentration-dependent application of ATRA and induction of BMP signalling (Metallo et al., 2008b); the authors reported that after 5 weeks of differentiation, 87% of cells expressed keratin 14.

Therefore, the aim of the set of experiments described in this chapter was to effectively combine the appropriate substrate and culture conditions to achieve an efficient production of functional epidermal progenitors from hESCs. Under these conditions, expression of basal keratins 5 and 14 is first detected in the peripheral cells, suggesting that these cells are the first to undergo differentiation. After 7 days of induction, cytoplasmic keratins 19, 5 and 14 can be detected, as well as the transmembranous desmosomal

marker DSC2. By day 25, large sheets of typical epithelial morphology could be observed. A few very large and flat cells could be seen at the edges of these putative epithelial sheets which were probably terminally differentiated cells that had lost replicative potential (Watt and Green, 1981). These cells were either only very dimly positive or negative for both keratin 14 and p63 expression. On the contrary, the central parts of the sheets displayed bright p63 positivity, indicating the presence of highly proliferative keratinocyte precursors. Following enrichment based on rapid adherence to type IV collagen, some rapidly adherent cells formed compact holoclone-like colonies that were brightly positive for p63 transcription factor expression, as well as keratin 14. These colonies were able to expand and form continuous large epithelial-like sheets, while maintaining their morphology and markers expression.

Flow cytometry analysis confirmed that pKPCs at passage 1 were homogenously positive for keratin 14 expression with 80.2% of the cells also positive for the α6 integrin subunit, a suggested marker for EpSCs (Li 1998), although this marker is not as effective as the ß1 integrin subunit in enrichment for human epidermal stem cells (Jones and Watt 1993; Jensen 1999). It is important to note that both α6 and ß1 integrins are highly expressed in undifferentiated hESCs (Xu et al., 2001), therefore only keratin 14 / α6 integrin double-positive cells can be considered putative keratinocyte-like cells. Homogenous keratin 14 expression was sustained after 5 passages, as indicated by a repeated flow cytometry analysis with a similar expression of α6 integrin (77.7%).

Previously, a limited proliferation potential of hESC-derived keratinocytes was reported (Aberdam et al., 2007, Green et al., 2003, Ji et al., 2006, Metallo et al., 2008b), and it was suggested that these cells represent an incomplete or abnormal form of p63-positive lineage development (Dabelsteen et al., 2009). Therefore in order to assess the proliferation potential and functionality of pKPCs produced, two assays which examine metabolic components that are necessary for cell growth, namely the CFE and MTT assays, were carried out. When CFE of pKPCs (6.3±1.3) was compared to that of NHKs (10.6±0.6), statistical analysis returned a value of p=0.0453. This is considered significant at 95% confidence interval (p<0.05). However as the value approaches the critical P value, it can be concluded that the clonogenic capacity of pKPCs closely resembles that of NHKs. Cell viability and proliferation was measured by the MTT assay and indicated that the O.D. for pKPCs at day 25 of differentiation was 0.16±0.03. When compared to NHKs (0.24±0.03), the difference was found to be statistically insignificant, suggesting a high proliferative capacity of epidermal progenitors produced under these conditions. The results of both assays suggest that pKPCs obtained on HDF-ECM under conditions described are functional, highly proliferative cells with a robust clonogenic capacity.

6.4.4 Conclusion and future work

In conclusion, the microenvironment provided by HDFs, in combination with BMP4 and ATRA, appears to encourage differentiation of hESCs towards highly proliferative keratinocyte precursors. These pKPCs retain

their morphological and immunocytochemical characteristics for up to 5 passages. These results are in accordance with the notion that cooperative development of the ectodermal and mesodermal layers during embryonic development is achieved through reciprocal signalling between these tissues (Sengel, 1976). In the coordinated ectodermal-mesodermal interactions, the ECM provides structural supports and directs a variety of cellular functions, proliferation, migration and differentiation.

Nevertheless, further functional studies are required to validate the potential of these pKPC cells. Of particular importance would be to see how these cells would behave when seeded on DED. In a previously published mouse model, reconstitution of fully functional epidermal layers as well as formation of a dermal compartment underneath was achieved (Coraux 2003). However, due to the relative inefficiency of the differentiation protocol and consequent heterogeneity of the epidermal cell population obtained, a continuous epidermis was not observed and only ~20% of the skin was well organized. The differentiation protocol described in this chapter, however, produced a pure population of keratin 14-positive cells and it is expected that DED would provide a favourable environment and further epidermal-stimulating cues for pKPCs to form continuous epidermis resembling the normal skin epidermis. Under such conditions pKPCs should be able to undergo terminal differentiation and stratification and arrange into distinct layers of cells at progressive stages of differentiation: with least differentiated cells in the basal layer and terminally differentiated cells on the surface.

CHAPTER 7 DISCUSSION OVERVIEW AND FUTURE DIRECTIONS

7.1 THE PROMISE AND PROSPECTS OF PLURIPTENT STEM CELLS FOR RDEB CELL THERAPY

Although the molecular basis of RDEB is reasonably well understood, effective treatment is still unavailable (Uitto et al., 2010). One potential treatment involves engineered allografted skin from normal individuals, which is one of the well-established cell therapies for severe burn victims (Pham et al., 2007). However, the allografted skin will eventually be immunologically rejected due to donor mismatch. Autologous skin cultures can also be generated from small pieces of healthy skin isolated from burn patients, and are commonly used for enhancing wound healing in instances of severe burns (Pham et al., 2007). However, this method cannot be implemented in patients with RDEB, because functional type VII collagen protein is markedly reduced or absent from all cells of affected individuals, i.e. autologous cells still retain the inherent susceptibility to skin fragility. Because the primary pathology of RDEB is due to a single gene defect, a "cure" would rely upon on the normal production of type VII collagen. Therefore, several current research approaches aim to restore collagen VII, including cell, gene and recombinant protein therapy (Mavilio et al., 2006, Titeux et al., 2010, Ortiz-Urda et al., 2003, Chen et al., 2002, Gache et al., 2004, Remington et al., 2009), and, more recently, bone marrow transplantation (Wagner et al., 2010a, Tolar et al., 2011a). For an optimal

approach in treating RDEB, keratinocytes should perhaps be the main cell type targeted, because it is the keratinocytes that produce the majority of type VII collagen in the skin *in vivo* (Regauer et al., 1990). That said, correction of the BM defect by genetically corrected fibroblasts alone has been demonstrated in a mouse model (Ito et al., 2009). With regards to keratinocytes, however, long-term success of the treatment is limited by the low proliferative capacity of keratinocytes, which renders them ineffective as targets for *ex vivo* gene therapy based on homologous recombination and by the low efficiency of targeting the keratinocyte stem cell pool. This might be a difficult task to accomplish *in vivo* because the pool of stem cells in normal skin is relatively limited, and, therefore, is likely to be reduced in RDEB skin due to constant sub-epidermal blistering and disruption of BM. Thus, one goal in developing a sustainable treatment for this disease would be to establish a robust approach for the derivation of keratinocyte stem cells or epidermal progenitors.

With this in mind, pluripotent stem cells can be considered the most promising cell type. The original derivation of hESCs by Thomson *et al.* (1998) stimulated a flurry of research focused on the potential for derivation of clinically useful cell types from hESCs, such as sensory neurons, skeletal muscle cells, insulin-producing pancreatic-like cells and others (Zheng et al., 2006, Puceat, 2008, D'Amour et al., 2005, Segev et al., 2004). These differentiation studies culminated in the first clinical trial using hESCs targeting spinal cord repair by transplantation of hESC-derived oligodendrocytes (Alper, 2009).

The discovery that somatic cell types can be reprogrammed to a pluripotent state (Takahashi et al., 2007) has encouraged a new wave of pluripotent stem cell research with the promise of obtaining iPSCs as a less ethically contentious alternative to hESCs. The reprogramming of RDEB-specific somatic cells into iPSCs is a promising new approach to establish human models for studying disease mechanisms, testing drugs, and developing cell therapies. Furthermore, patient-specific RDEB iPSCs can be gene-targeted by homologous recombination to correct the *COL7A1* gene defect.

An intriguing alternative for skin diseases is the readily available source of spontaneously corrected skin cells in the form of revertant mosaicism (Almaani et al., 2010, Jonkman et al., 1997, Pasmooij et al., 2010, Pasmooij et al., 2007, Pasmooij et al., 2005). Occasionally, patients with different forms of EB and other skin diseases display patches of phenotypically normal skin where second-site mutations have resulted in restoration of protein expression. Somatic reprogramming of keratinocytes from these revertant patches and subsequent generation of iPSC-derived epidermal progenitors has the potential to provide an unlimited source of patient-specific autologous cells that can eventually be used for skin grafting or transplantation without the need for conditioning or immunosuppression or any additional correction of the skin BM genetic defect.

Therefore, mastering manipulation of pluripotency would provide a proof of concept that pluripotent stem cell derivatives may be a source of keratinocytes for RDEB cell therapy.

7.2 SUMMARY OF RESULTS

The studies presented in this thesis have been concerned with, firstly, devising clinically- relevant conditions for culture and/or derivation of pluripotent stem cells and, secondly, with the attempted derivation of epidermal progenitor-like populations of cells from hESCs.

7.2.1 Studies of pluripotent cell culture and derivation

Feeder-based culture, as currently done in most laboratories, requires manual passaging and makes maintenance of human pluripotent stem cells in an undifferentiated state extremely labour-intensive and time consuming. To overcome this issue, the KCL-002 hESC line was adapted to feeder-free growth prior to commencement of differentiation studies.

Under feeder-free conditions, robust expression of mRNA transcripts of pluripotency-associated Nanog and Sox-2 genes was detected. Immunocytochemical analysis of KCL-002 colonies also revealed consistent expression of pluripotency-associated markers. Undifferentiated cells were able to undergo directed differentiation through embryoid body formation *in vitro*, and proved to possess germ layer competence during teratoma assays *in vivo.* Thus, Chapter 2 of this study demonstrated that hESCs can be successfully adapted to growth under defined feeder-free culture conditions and expanded using enzymatic treatment, allowing for scalability, which in turn permits repeated differentiation attempts and their evaluation.

In the work described in Chapter 3, I attempted to achieve somatic cell reprogramming using whole protein extracts of the KCL-002 hESC line. Unfortunately, despite observing colonies with iPSC-like morphology and SSEA-4 pluripotency marker expression, I was not able to obtain a fully-reprogrammed cell population. The possible explanations for this negative result are discussed in more detail in Chapter 3, and it remains to be determined if indeed the generation of human iPSCs can be achieved using this methodology.

7.2.2 Studies regarding the derivation of epidermal progenitors from hESCs

Chapter 4 of this thesis presented data showing that, while a complex microenvironment such as DED was able to induce formation of some epidermis-like structures from undifferentiated hESCs, the conditions were insufficient to promote the development of a continuous pluristratified epidermis. It was, therefore, concluded that to achieve the development of a fully-formed epidermis, a highly proliferative population of epidermal progenitors should be first obtained from hESCs. Consequently, Chapters 5 and 6 described the evaluation of various differentiation protocols that could serve this purpose.

In data presented in Chapter 5, comparison of different methods relying on SDIA exerted by PA6 stromal cells was drawn, and the results suggested that indirect co-culture using Transwell systems was most effective in

inducing epidermal differentiation of hESCs. However, pKPCs obtained displayed a limited proliferative potential which would severely hinder further clinical application of these cells.

These observations are consistent with previous differentiation studies, showing that hESC-derived keratinocytes generated fragmented colonies formed by scattered noncohesive cells and, more importantly, possessed a much lower proliferative potential compared to NHKs (Green *et al.*, 2003; Aberdam *et al.*, 2007; Iuchi *et al.*, 2006; Ji *et al.*, 2006; Metallo *et al.*, 2008). The differentiation protocol described in Chapter 6, on the other hand, showed that the epidermal progenitors produced had a very high proliferative capacity, and, therefore, could potentially be used as a continuous source of keratinocytes in future therapies for RDEB. It appears that the combination of HDF-ECM and synergetic effects of BMP-4 and ATRA can generate and sustain a relatively less differentiated population of hESC-derived epidermal stem cells or long-live progenitors, compared to the minimally proliferating hESC-derived keratinocytes that have been previously described. Indeed, the observed holoclone-like colonies and clonogenic efficiency assay would suggest that pKPCs obtained under the conditions described were enriched in holoclone-forming cells, as opposed to the meroclone- and paraclone-forming population of more differentiated transient amplifying progenitors (De Luca et al., 2006).

Collectively, the data support and further emphasise the importance of recapitulating epidermal morphogenesis, in particularly the inductive

effects of mesenchyme (Sengel, 1976), *in vitro* in order to achieve efficient and reproducible epidermal differentiation from hESCs.

7.3 RECONCILIATION OF DATA WITH THE PUBLISHED LITERATURE

In the section that follows, I assessed the integration of data presented in this thesis with that of published literature in the relevant field.

7.3.1 Evidence for epidermal cell lineage derivation from hES cells

Several studies have demonstrated the possibility for derivation of keratinocytes frm hESCs (Aberdam et al., 2007, Green et al., 2003, Ji et al., 2006, Metallo et al., 2008b), albeit with varying degrees of efficiency, reproducibility and clinical potential.

Recently, the first pre-clinical trial was conducted which utilized epidermal progenitors obtained after 40 days of treatment with BMP-4 and ATRA (Guenou et al., 2009). These hESC-derived keratinocytes appeared capable of forming a pluristratified epidermis, both *in vitro* and *in vivo,* as xenografts in immunodeficient mice. The resultant human epidermis was shown to persist for at least 3 months, that is, at least three full epidermal renewal cycles. These observations imply that initiation of studies and possible clinical trials into the repair of human epidermal defects using hESC-derived keratinocytes is not inconceivable.

7.3.2 Evidence for generation of RDEB-specific iPSCs

Recently, successful derivation of RDEB-specific iPSCs has been reported (Itoh et al., 2011, Tolar et al., 2011b). While one of these studies suggested that these RDEB-iPSCs could be used to ameliorate the disease through differentiation into a haematopoietic lineage (Tolar et al., 2011b), the other study showed that RDEB-iPSCs can undergo a directed differentiation into fully-functional keratinocytes (Itoh et al., 2011), providing a proof-of-concept for potential therapeutic application of these cells. It is worth noting, however, that the percentage of keratin 14-positive cells under differentiation conditions tested in that study was approximately 70% (Itoh et al., 2011), which is significantly lower than that achieved with the differentiation protocol described in Chapter 6 of this thesis. More importantly, both of these published studies relied on retroviral transduction for iPSC generation (Itoh et al., 2011, Tolar et al., 2011b), and a variable viral transgene silencing was reported (Itoh et al., 2011). Therefore, further efforts should be directed towards establishing an efficient and reproducible protocol for RDEB-specific iPSCs using non-integrating reprogramming methods. In addition, a parallel comparison with hESC-derived epidermal progenitors with those derived from iPSCs would be desirable.

7.4 FUTURE STUDIES

7.4.1 Short-term goals

Immediate studies should be directed to evaluate the capacity of epidermal progenitors obtained in the differentiation protocol described in Chapter 6 to form continuous pluristratified epidermis with appropriate marker expression in organotypic cultures.

In addition, examination of stage-specific expression of epidermal marker genes, particularly p63 transcription factor, would also augment the data.

7.4.2 Long-term goals

In order to translate the differentiation approach described in Chaper 6 of this thesis into a clinical setting, scalability of the differentiation process would be required. Automation culture of pluripotent stem cells using bioreactors (Come et al., 2008) and the full automation of cell culture on a CompacT SelecT platform capable of producing two billion cells every 2 days (Thomas et al., 2009) opens the prospects for large-scale production of hESCs. In addition, similar platforms could be designed for differentiation protocols, thus allowing for gain in scalability at these steps also.

7.5 IMPLICATIONS OF THE PRESENT STUDY AND FINAL CONCLUSIONS

Identification of a differentiation protocol that coaxes pluripotent stem cells at an undifferentiated stage towards an epidermal lineage, with the resulting cell population exhibiting characteristics similar to those of basal keratinocytes, opens exciting avenues for a potential cell therapy for RDEB, as well as skin grafting in general.

Despite high demand and the available technology, an adult keratinocyte cell bank has not been established. That said, adult keratinocytes might not be the most appropriate candidate for cell banking, since the formation of a reconstructed epidermis is highly dependent on the age of donors (Youn et al., 2004) and the number of cell passages (Fortunel et al., 2003), which is generally high in allogeneic cultures. Furthermore, batch-to-batch variability would be unavoidable as cells would be obtained from individuals with different genetic backgrounds. On the other hand, it might indeed be possible to explore the potential of pluripotent stem cells- either of embryonic origin or produced by somatic reprogramming- and establish a large bank of clinical-grade epidermal progenitors, capable of forming a well-differentiated pluristratified epidermis fulfilling the needs of hundreds of thousands of patients who may benefit from it. From the studies of BM transplantation in RDEB (Kiuru et al., 2010, Wagner et al., 2010a), these epidermal progenitors could be administered topically as well as systemically to ameliorate skin disease.

There are, however, some issues which have to be addressed in order to bring the application of these cells closer to the clinical setting. One issue is the potential immunogenicity of the epidermal progenitors, although one may expect cells displaying characteristics of foetal or embryonic skin to have a much better tolerance than adult keratinocytes. The possibility of a bank of pluripotent stem cell lines of a wide variety of haplotypes may eventually provide a definitive answer.

Another important concern regarding the potential clinical utility of pluripotent stem cells is the risk of teratoma formation. Although a teratoma is not in essence malignant, its natural propensity to grow makes it potentially dangerous when implanted into an individual and, as such this would be unacceptable for an approved cell therapy. Despite confirmation of absence of pluripotency-associated markers in the epidermal progenitors obtained, the possibility that a rare cell has retained its pluripotent characteristics, or that grafting itself can cause dedifferentiation, cannot be excluded. However, the superficial placement of the graft for epidermal cell therapy would allow for a relatively easy continuous control by monitoring appearance of the tissue and its surgical resection in case of an emerging problem.

In conclusion, it is worth noting that most cell types that normally participate in the formation of the dermis and epidermis can either already be obtained through *in vitro* differentiation from pluripotent stem cells or would likely migrate from the host into a graft. However, differentiation protocols for specialized glands and hair follicles remain to be designed.

Therefore, a future can be foreseen when reconstructive medicine will make use of composite grafts integrating several different cell types with biomaterial technology.

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